MOLECULAR INSIGHT INTO CELL SURFACE NUTRIENT TRANSPORTER QUALITY CONTROL AND DOWNREGULATION IN *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

Plasma membrane integrity is paramount to cell viability. The separation between the extra- and intracellular environment is established by the plasma membrane and the plethora of proteins embedded within it. Nutrients that are unable to freely diffuse across the plasma membrane must be transported. Transportation is a highly regulated process. The proteins that facilitate nutrient transport, plasma membrane nutrient transporters, are multispanning integral membrane proteins, which utilize the energy of ion gradients to transport nutrients into the cell. Metabolic demands of the cell regulate the abundance of plasma membrane nutrient transporters by influencing new protein synthesis or protein degradation.

Appropriate downregulation and vacuole degradation of plasma membrane nutrient transporters is imperative to maintain cellular homeostasis. Downregulation of nutrient transporters has been observed both on a global, cell-wide scale, targeting many different transporters congruently, and on a protein-specific basis, resulting in a single transporter's downregulation. In *Saccharomyces cerevisiae*, downregulation is facilitated by the E3 ubiquitin ligase Rsp5. For specific downregulation of a nutrient transporter to occur, Rsp5 must recognize the correct substrate before ubiquitin conjugation. How this is achieved is an open question in the field.

Identification and subsequent downregulation of damaged cell surface nutrient transporters require Rsp5 to properly distinguish between a damaged and nondamaged protein. It has been observed that Fur4, the high affinity uracil transporter, is efficiently downregulated in response to both peroxide and heat stress, but the underlying mechanism was unknown. Utilizing the crystal structure of Mhp1, a bacterial homolog of Fur4, an intrinsic protein-fold sensing domain was identified and termed the Loop Interaction Domain (LID). Through extensive mutational analysis, it was discovered that the LID of Fur4 functions as a built-in chaperone: The LID directly relays the folded status of Fur4 to the ubiquitin machinery of the cell by exposure or sequestration of a degron. The data presented here resulted with the discovery of the LID-degron mode of degradation, which is a conformational model explaining both quality control and substrate-dependent downregulation and how Rsp5 is able to identify specific substrates.

Dedicated to my loving wife, Leslie. Without her continued support, none of this would have been possible.

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LIST OF ABBREVIATIONS

Abbreviations	Defining Term
APC	Amino acid/Polyamine/Organocation
ART	Arrestin-Related Trafficking Adaptors
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
ESCRT	Endosomal Sorting Complexes Required for Transport
HOPS	Homotypic Fusion and Vacuole Protein Sorting
ILV	Intraluminal Vesicles
LID	Loop Interaction Domain
MVB	Multivesicular Body
PI-3P	Phosphatidylinositol 3-Phosphate
PKA	Protein Kinase A
PY	PPxY Motif
SNARE	Soluble *N*-Ethylmaleimide-Sensitive-Factor Attachment Protein Receptor
TOR	Target of Rapamycin
TORC1	TOR Complex 1
TCAs	Tricyclic Antidepressants

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CHAPTER 1

INTRODUCTION

Eukaryotic cells continually take up nutrients from their extracellular environment through cell-surface nutrient transporters. Intracellular concentration of nutrients depends upon the extracellular levels of nutrients available for transport and the number of cognate transporters present at the plasma membrane. The cytoplasmic levels of these nutrients are a key factor that determines the activity of metabolic pathways in the cell. Therefore, the underlying principles of nutrient transporter regulation (synthesis, trafficking, and degradation) and how this regulation is influenced by the metabolic needs of the cell are central questions in cell biology. Because the basic metabolic pathways and the nutrient transporters providing the necessary substrates are conserved among eukaryotes, we utilized the tractable model organism *Saccharomyces cerevisiae* to study the regulation of nutrient transporter systems (Tugendreich et al., 1994). Unless otherwise noted, the subsequent background/introduction will focus primarily on the trafficking pathways in yeast that are involved in regulating nutrient transporters.

The APC (Amino acid/Polyamine/Organocation) Transporter Superfamily

Members of the APC transporter superfamily function as solute:cation symporters and solute:solute antiporters. In general, the reaction catalyzed by these transporters is: Substrate(Jund and Lacroute)+ Ion(Jund and Lacroute)→ Substrate(in)+ Ion(in) (Schweikhard and Ziegler, 2012). Proteins within the APC superfamily have been found in organisms ranging from archaea to mammals and thus are considered ubiquitous. APC transporters have 12 transmembrane domains (Jack et al., 2000; Wong et al., 2012) and can either be generalist or specialist with regard to substrate specificity. Structure determination of APC superfamily members, such as the crystal structure of the bacterial leucine transporter LeuT, have given detailed insight into the mechanism not only of nutrient import but also into the function of related permeases, such as the serotonin transporter that plays a key role in brain activity of higher mammals (Krishnamurthy and Gouaux, 2012). The diversity of molecules that yeast imports mirrors the diversity of transporters that facilitate their uptake (André, 2004). Transporters vary from general transporters, such as Gap1, which is able to transport all naturally occurring L-amino acids, to specialized transporters like Fur4, the high affinity uracil transporter (Jauniaux and Grenson, 1990; Jund and Lacroute, 1970). APC transporters not only import metabolic substrates but also salts and metals. Yeast APC transporters are proton-driven, requiring a proton gradient across the plasma membrane for transport of a substrate (Jack et al., 2000; Shimamura et al., 2010). The proton gradient is maintained by the P-type

H^+ ATPase, Pma1p, which utilizes ATP hydrolysis for pumping protons out of the cell in a predicted one-to-one stoichiometry of ATP: H^+ (Ambesi et al., 2000). Pma1 is not only responsible for maintaining the plasma membrane electrochemical proton gradient but also required for maintaining proper cellular pH (Ambesi et al., 2000). The electrochemical proton gradient ensures that nutrient transport occurs unidirectionally across the plasma membrane.

In recent years, the crystal structures of two bacterial APC superfamily members, LeuT and Mhp1, have been determined (Krishnamurthy and Gouaux, 2012; Shimamura et al., 2010). Both are homologs of the yeast nutrient transporters such as Mup1 and Fur4, which import methionine and uracil, respectively. The structure determination of Mhp1 and LeuT has given great insight into the transport mechanism. Based on structure determination of different transport states, a so-called 'alternating access transport model' has been proposed to explain the transport function of this class of permeases (Shimamura et al., 2010) (see Fig. 1.1). The transport cycle is proposed as follows: (1) In the initial outward-facing open state (also referred to as the ground state), the substrate and proton binding pockets are exposed to the extracellular environment. (2) The binding of both a proton and substrate induces a conformational change that occludes the binding pockets. (3) The protein undergoes a large conformational change, results in an inward-facing, occluded state. (4) A shift in protein conformation results in the inward-facing, open state where the substrate and proton are released into the cytoplasm (Krishnamurthy and Gouaux, 2012; Shimamura et al., 2010). Once the final step is complete, the

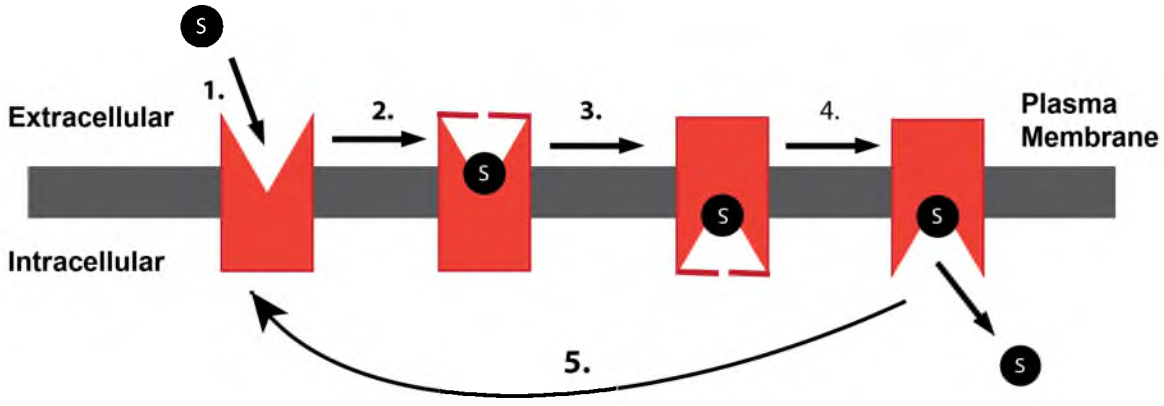

Figure 1.1 Cartoon image depicting the alternating access transport model of APC superfamily transporters

1. Outward Open: Substrate has access to binding pocket
2. Outward Occluded: Substrate is bound and blocked from diffusing away
3. Inward Occluded: Substrate is bound and blocked from diffusing away
4. Inward Open: Substrate is free to diffuse into cytoplasm
5. Transporter reverts back to outward open conformation

protein can revert back to the initial ground state, allowing further rounds of substrate transport. (Refer to Fig. 1.1 for a model of the 'alternating access transport model.')

Not only do nutrient transporters function in nutrient import, but there is a subset that has been identified to act as transceptors. Transceptors are defined as nutrient transporters or nutrient transporter-like proteins that have the added function of a signaling receptor (Thevelein and Voordeckers, 2009). In yeast, there are several examples of transceptors, but two examples stand out: the general amino acid transporter, Gap1, and Ssy1, a component of the yeast cell surface nutrient amino acid sensing system (Kriel et al., 2011; Thevelein and Voordeckers, 2009). Ssy1 senses extracellular amino acid concentrations and in turn initiates signals that modulate expression of nutrient permease genes (Wu et al., 2006). The model of Ssy1 activity involves a conformational switch dependent on the concentration ratio of intra- vs. extracellular amino acids (Wu et al., 2006). Gap1, an active nutrient transporter transceptor, signals and activates the protein kinase A (PKA) pathway in the presence of substrate. In general, the PKA pathway coordinates the expression of genes required for cell growth. Thus, transceptors link the function of nutrient transport with the downstream regulation of cellular metabolic pathways.

The connection between the metabolic demands of the cell and the active scavenging of nutrients from the extracellular environment through the activity of cell surface nutrient transporters is an important and fundamental cellular process. Cells regulate nutrient fluxes by modulating the cell surface

concentration of nutrient transporters. There are three distinct methods for this regulation: (1) up- or downregulation of transporter synthesis, (2) nutrient transporter relocalization (plasma membrane or internalized pool), and (3) transporter degradation. Both nutrient transporter relocalization and degradation are responses that can act quickly according to cellular demands. Regulating gene expression is a slower cellular response than the simple trafficking of a nutrient transporter to or from the plasma membrane.

Out of the three methods of regulating nutrient transports, the degradation of nutrient transporters and the underlying mechanism required for specific nutrient transporter removal from the plasma membrane was chosen for further study. To investigate how the cell regulates nutrient transporter turnover, the model cargo of Fur4 and Mup1 were both utilized. The trafficking events required for an APC transporter such as Fur4 to become plasma membrane localized and eventually degraded will be discussed in the following chapter. (Refer to Fig. 1. 2 for a schematic overview of yeast trafficking pathways.)

Trafficking of the Uracil Transporter Fur4

Translation at the endoplasmic reticulum. Fur4 begins its life cycle with translation and translocation/threading into the membrane of the endoplasmic reticulum (ER), the initial entry point to the secretory pathway. *Saccharomyces cerevisiae* has two distinct pathways for protein insertion into the ER: cotranslational and posttranslational. Insertion into either the ER lumen or membrane requires the protein-conducting membrane channel termed the translocon. (Refer to Table 1.1 for translocon-associated proteins.) Post-

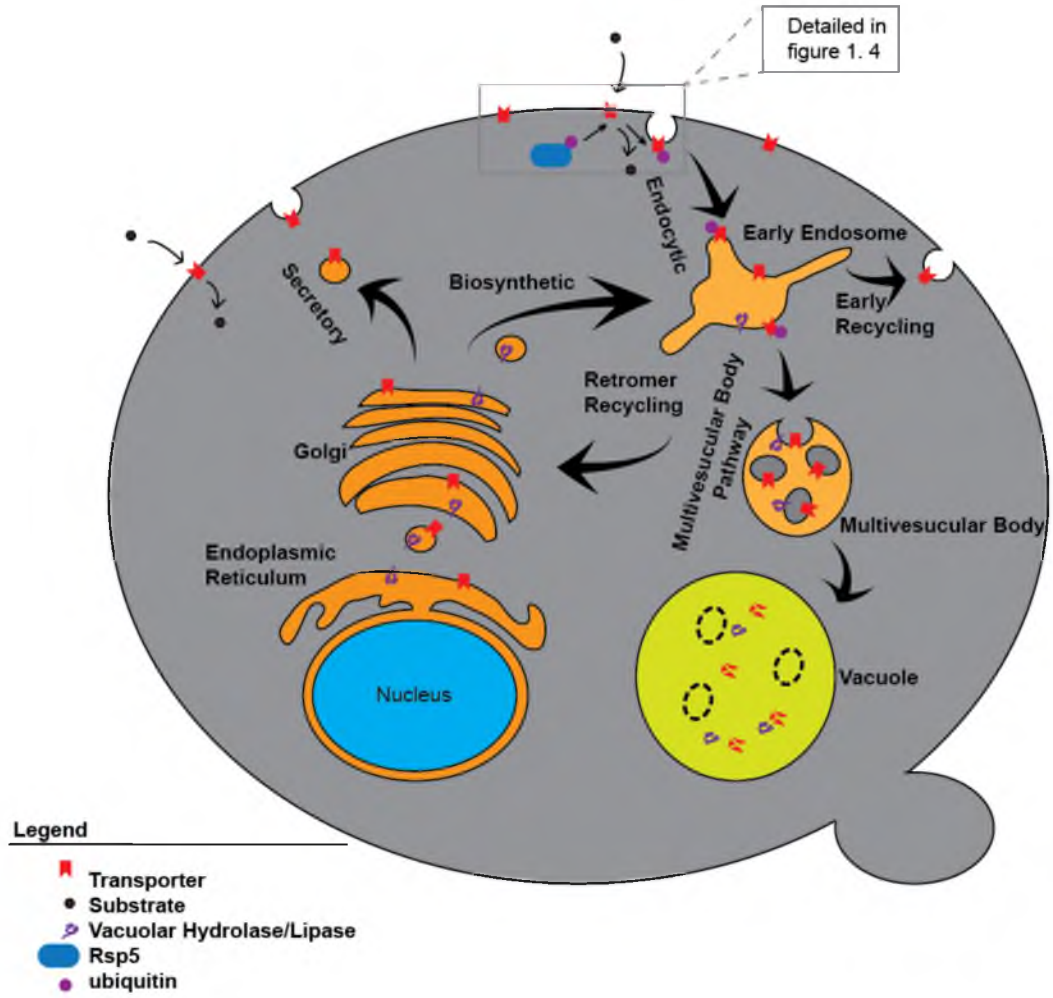

Figure 1. 2 Schematic overview of trafficking pathways in *Saccharomyces cerevisiae*.

Table 1.1 Translocon proteins involved in ER insertion

Protein Complex	Protein	Function	Reference
Translocon	Sec61	Central pore formation	(Park and Rapoport, 2012)
	Sss1	Structural clamp of Sec61	(Esnault et al., 1994)
	Sbh1	Stabilizes the Sec61 and Sss1 interaction	(Finke et al., 1996)

translational insertion requires cytoplasmic molecular chaperones to ensure that the completed polypeptide remains soluble and free in the cytoplasm to interact and pass through the translocon (Zimmermann et al., 2011). Cotranslational insertion into the ER requires translation to be stalled, which is accomplished by the recognition of a signal sequence. The signal recognition particle then facilitates ribosomal targeting and docking to the translocon (Akopian et al., 2013; Jiang et al., 2008). After the ribosome interacts with the translocon, translation resumes and the nascent polypeptide moves into the central pore of the translocon. If the peptide being translated is a single or multiple pass transmembrane protein, the translocon must release the transmembrane domains through a lateral gate into the ER membrane (Zimmermann et al., 2011). The translocon can hold up to four transmembrane domains within the central pore before release into the membrane (Zimmermann et al., 2011). Fur4, being a multiple transmembrane domain containing protein, is predicted to be inserted cotranslationally into the ER membrane. Because Fur4 has 12 predicted transmembrane domains, translation must occur with at least three steps, if the translocon holds the maximum of four transmembrane domains

at a time. It is not explicitly known how Fur4 is inserted into the ER membrane, but a likely scenario is as follows: Translation occurs of the first four transmembrane domains, then pauses while they are held within the translocon and then inserted into the lipid bilayer through the lateral gate. Translation then resumes and the second group of four transmembrane domains is inserted. This process would continue until the entire protein is translated and inserted into the ER membrane.

Trafficking from the ER to the trans-Golgi. Once translation and insertion are completed, Fur4 must fold correctly and pass beyond the ER quality control system before ER exit is allowed. (ER quality control will be discussed later in this chapter.) Properly folded Fur4 undergoes packaging into vesicles destined for the cis-Golgi. The mechanism of cargo selection and subsequent vesicle packaging in yeast is not well understood. Vesicle-mediated ER to cis-Golgi trafficking is termed 'anterograde transport,' and is mediated by the coat protein complex II (COPII) (Tang et al., 2005). Conversely, 'retrograde transport' is Golgi to ER trafficking and is COPI-mediated (Gaynor et al., 1998). Vesicles that bud from the ER membrane first traffic to an intermediate compartment termed the ER-Golgi intermediate compartment (ERGIC), as predicted from the stable compartment model of anterograde membrane traffic (Appenzeller-Herzog and Hauri, 2006). The ERGIC is considered a sorting station for ER to Golgi cargo, and is the first location for retrograde transport back to the ER. Fusion of ER-budded vesicles with the ERGIC is a SNARE (soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor)-mediated process (Nichols and

Pelham, 1998). Cargos delivered to the ERGIC are either selected to return to the ER in a COPI-mediated process or allowed to stay with ERGIC as it matures into the cis-Golgi. Once Fur4 is delivered to the cis-Golgi, it is trafficked through the maturing Golgi cisterna towards the trans-Golgi. ER and Golgi are locations of protein glycosylation, but there is no evidence that Fur4 is glycosylated (André, 2004).

Trafficking from the trans-Golgi. Once located at the trans-Golgi, Fur4 can undergo packaging into vesicles destined for two distinct targets: the plasma membrane to function or the endosomal system for degradation. (Trans-Golgi to endosomal trafficking will be discussed later.)

The default pathway for Fur4 trafficking is from the trans-Golgi to the plasma membrane. Fur4, without a specific sorting signal, is packaged into vesicles delivered to the plasma membrane. The exact mechanism required for packaging and vesicle formation of trans-Golgi to plasma membrane bound vesicles is not well understood. Once transported and integrated into the plasma membrane, Fur4 functions to transport extracellular uracil into the cell.

Fur4 at the plasma membrane. Fur4 is known to be located within specialized lipid environments, termed lipid rafts. Lipid rafts are membrane microdomains comprised of sphingolipids and ergosterol that form puncta on the cell surface (Dupre and Haguenauer-Tsapis, 2003). It has been observed that Fur4 association with lipid rafts is important for efficient trafficking to the plasma membrane (Dupre and Haguenauer-Tsapis, 2003). Not only is Fur4 associated

with these lipid rafts, but with Pma1 and a host of other multispanning plasma membrane proteins as well (Dupre and Haguenauer-Tsapis, 2003).

Degradation of nutrient transporters, also referred to as downregulation, is initiated by ubiquitin-triggered endocytosis (Dupre et al., 2004; Lauwers et al., 2010; MacGurn et al., 2012). Ubiquitin is a highly conserved 76 amino acid regulatory protein that is covalently attached to target proteins. The process of ubiqutination requires the sequential activity of the ubiquitin system (reviewed in (Hershko and Ciechanover, 1998). Briefly, the ubiquitin system is comprised of three enzymes: a ubiquitin–activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase enzyme (E3). Protein ubiqutination requires three basic steps: (1) E1 uses the energy supplied by ATP hydrolysis to covalently attach the C-terminal carboxyl group of ubiquitin to its active site cysteine through a thioester linkage; (2) transfer of the ubiquitin conjugate from E1 to an E2 active site cysteine through a transesterification reaction; (3) E3 catalyzes the formation of an isopeptide bond between an accessible lysine of the substrate protein and the C-terminal glycine of E2-conjugated ubiquitin. Ubiquitin modifications occur either with single ubiquitins (mono-ubiquitination) or by the addition of a poly-ubiquitin chain. These chains are formed by the attachment of ubiquitin to any of the seven exposed lysine residues of the substrate ubiquitin. The role of mono- versus poly-ubiqutination as related to transporter downregulation is still a matter of debate, but it is commonly accepted that monoubiquitination is sufficient to trigger endocytosis of the transporter

(Stringer and Piper, 2011). Ubiquitination, like phosphorylation, is a reversible process that is facilitated by deubiquitinating enzymes.

Ubiquitination of Fur4 is facilitated by the E3 ubiquitin ligase Rsp5 (Lauwers et al., 2010). Rsp5, a member of the Nedd4 HECT (E6AP-type E3 ubiquitin-protein ligase) family of ubiquitin E3 ligases, contains a N-terminal C2 domain, three WW domains, and a C-terminal HECT domain (Ingham et al., 2004). The C2 domain is responsible for targeting Rsp5 to multiple membrane locations throughout the cell, including the plasma membrane and endosomal membrane (Dunn et al., 2004). The catalytic HECT domain of Rsp5 is responsible for addition of ubiquitin or lysine-63 polyubiquitin chains to substrates (Rotin and Kumar, 2009). Rsp5 not only functions as the key ubiquitin ligase of cell-surface nutrient transporters, but has a plethora of other cellular duties including regulating translation, influencing mitochondrial inheritance, and modifying chromatin (Ingham et al., 2004).

Rsp5-mediated ubiqutination initiates endocytosis of most plasma membrane nutrient transporters, but there is a complication for Rsp5 that arises from the vast diversity and complexity of plasma membrane transporters: How is Rsp5 able to determine which transporters require ubiquitin conjugation and which do not? Rsp5 binds to its targets via the WW domains, which interact with proteins containing PPxY (PY) motif (Belgareh-Touze et al., 2008). The caveat is that many cell-surface nutrient transporters do not exhibit a PY motif (Belgareh-Touze et al., 2008) and these transporters require adaptor proteins to recruit

Rsp5. Recently, a wide variety of adaptor proteins have been identified, called the ARTs (arrestin-related trafficking adaptors) adaptor protein family. These proteins have been implicated in targeting Rsp5 to specific substrates in a ligand- and stress-dependent manner (Lin et al., 2008; Nikko and Pelham, 2009). In yeast, the ART family has a predicted 10 members based on motif similarity with the Art1 arrestin motif, but only a few have been characterized (Lin et al., 2008). Art1 was shown to interact with Rsp5 via a PY motif, thereby recruiting Rsp5 to at least five different nutrient transporters (Lauwers et al., 2010). It is predicted that each ART protein has a specific set of nutrient transporters to which it binds and recruits Rsp5 for ubiquitination. The ART family is not the only known Rsp5 set of adaptor proteins: There are at least another six adaptor proteins known (Lauwers et al., 2010). (For an Rsp5, Adaptor, substrate map, refer to Fig. 1.3.) (For a schematic model of Rsp5 mediated ubiquitination, refer to Fig. 1. 4.)

Fur4 in the endocytic pathway. Upon ubiquitination, Fur4 is removed from the plasma membrane by clathrin-mediated endocytosis, a complex and tightly regulated process that has been studied in detail (for a review see Weinberg and Drubin, 2012). After clathrin uncoating, endocytic vesicles are able to fuse in a SNARE-mediated event with the early endosome, a hub for cellular protein transport. Proteins delivered to the early endosome can be packaged for transport to three different cellular locations: the plasma membrane, the Golgi apparatus, or the vacuole.

The recycling back to the plasma membrane is termed ‘early recycling’ and is the least well known within the yeast model, but has been characterized in

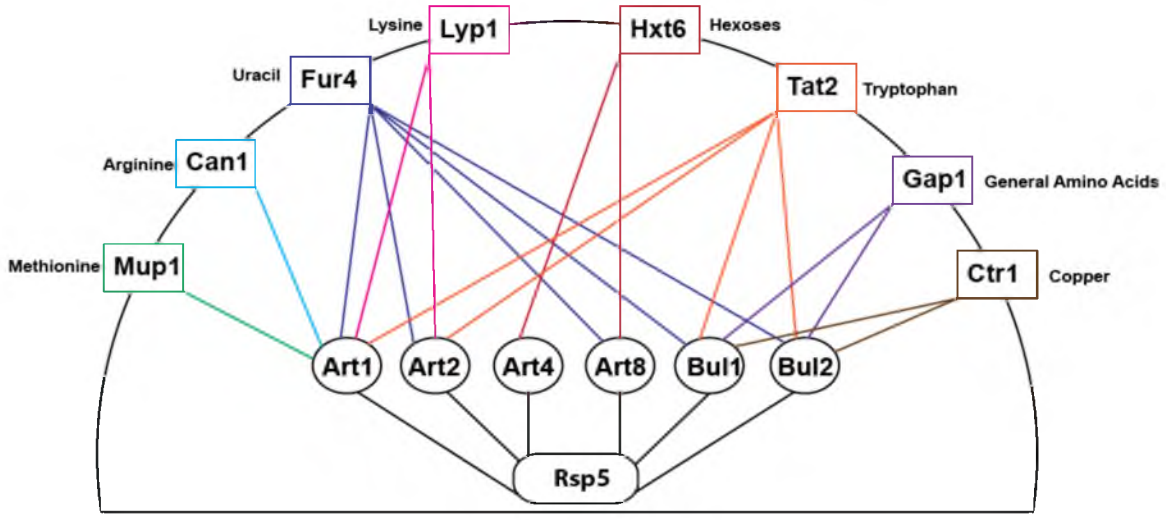

Figure 1. 3: Interaction map depicting Rsp5, adaptor proteins, and known nutrient transporters that interact with the adaptors. (Reviewed in Lauwers et al., 2010)

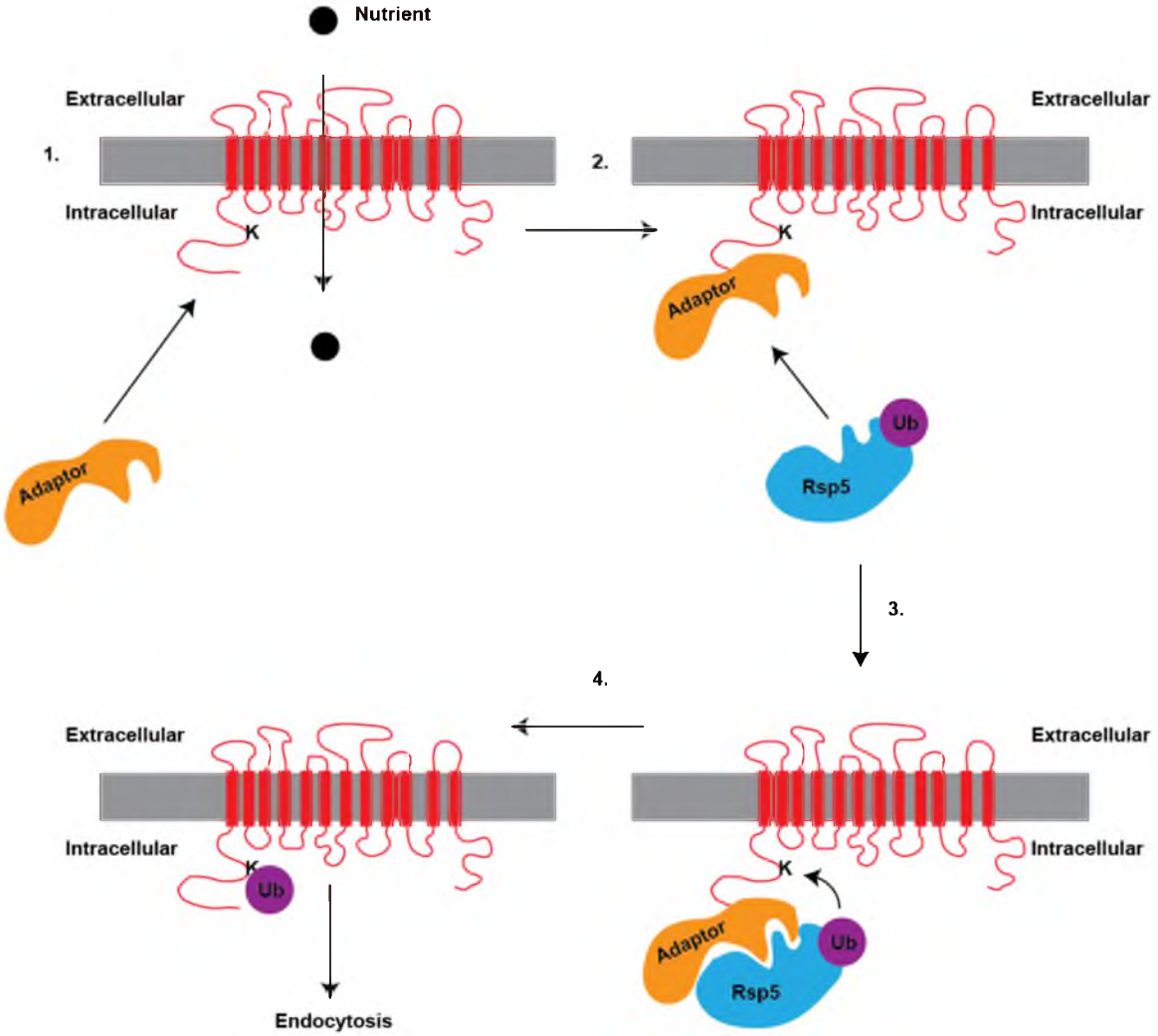

Figure 1.4 Basic model of substrate dependent downregulation.

1. Transporting nutrients recruits an adaptor protei to the transporter
2. The adaptor in turn recruits the E3 ligase Rsp5
3. Rsp5 then ubiquitinates exposed lysine residues
4. Ubiquitination results in endocytosis

the mammalian system. (Refer to Fig. 1.2.) The early recycling pathway is considered the default pathway for transmembrane proteins and requires no specific sorting signal (Babst, 2005; Seaman, 2005; Tanno and Komada, 2013). Cargos delivered to the early endosome go through a constant process of deubiquitinating and ubiquitination. Ubiquitination of transmembrane proteins at the early endosome is a signal for degradation and will be explored in more detail shortly. Deubiquitination, on the other hand, would allow the protein to traffic by the early recycling pathway back to the plasma membrane. The cyclic deubiquitinating and ubiquitination is facilitated by an early endosomal-localized protein complex that contains the E3 ligase Rsp5 and the deubiquitinating enzyme Ubp2 and is mediated by a physical interaction with the cytoplasmic protein Rup1 (Kee et al., 2005). Though it is believed that Fur4 is able to traffic by this early recycling pathway, it has not been explicitly demonstrated.

The early endosome recycling to the trans-Golgi or the late recycling pathway requires the coat protein retromer complex (Seaman, 2005). Entry into the retromer pathway requires specific sorting signals that are recognized by sortin nexins (Cullen and Korswagen, 2012). Fur4 does not exhibit any known sorting signal for this pathway.

The third trafficking route from the early endosome delivers protein cargos to the vacuole, the hydrolytic compartment responsible for protein and lipid degradation. (Refer to Fig. 1.2.) Cargos trafficked from the early endosome to the vacuole have two distinct delivery locations: the limiting membrane of the vacuole or the lumen of the vacuole. Cargos destined for the lumen of the

vacuole require the sorting signal of ubiquitin. Ubiquitinated cargos are then recognized and sorted into intraluminal vesicles (ILV) within the maturing endosome. The term for an endosome with ILVs is a multivesicular body (MVB). Once the MVB or late endosome fuses with the vacuole, all cargos within ILVs are delivered to the lumen of the vacuole. In contrast, transmembrane proteins delivered to the early endosome without an ubiquitin sorting signal stay at the limiting membrane for delivery to the limiting membrane of the vacuole. The trafficking pathway of cargos from the early endosome to the lumen of the vacuole is termed the MVB pathway, and requires the function of the Endosomal Sorting Complexes Required for Transport (ESCRTs). ESCRTs facilitate the packaging of cargos into ILVs and the formation of MVBs (Babst, 2005; Babst, 2011).

Four discrete ESCRT complexes and the Vps4 complex are required for MVB formation. (Refer to Fig. 1. 5 for an epistasis model of the ESCRT system.) (See Table 1. 2 for ESCRT complex components and function.) The endosome is enriched in phosphatidylinositol 3-phosphate (PI-3P), a lipid produced by the phosphatidyl-inositol kinase Vps34 (Schu et al., 1993). ESCRT-0 is recruited to endosomes by binding to the head group of PI-3P (Schmidt and Teis, 2012). ESCRT-0 initiates cargo sorting by recognizing ubiquitinated transmembrane proteins at the endosome (Henne et al., 2011). After ESCRT-0 binds to the endosome it recruits ESCRT-I to the endosomal membrane through a direct protein-protein interaction (Henne et al., 2011). ESCRT-I like ESCRT-0, functions in sorting ubiquitinated cargo, but also recruits the ESCRT-II complex

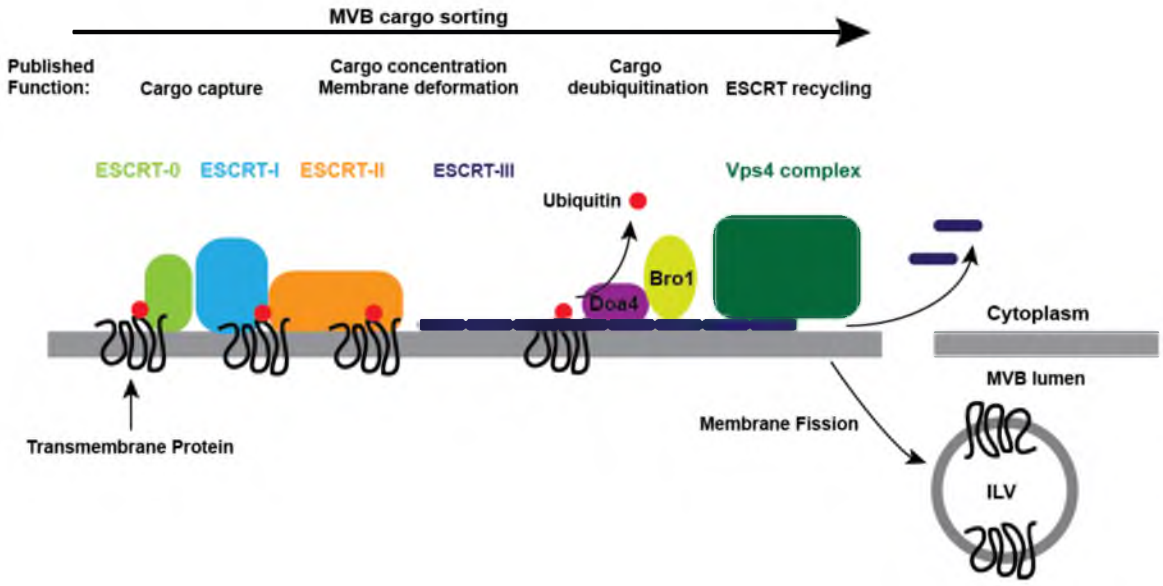

Figure 1.5. Epistasis model of the ESCRT system (Refer to Table 1.2 for a list of ESCRT complex components and functions).

Table 1. 2. MVB pathway-associated proteins and protein complexes (Reviewed in Hurley, 2010)

Protein Complex	Protein	Function and or Binding Partners
ESCRT-0		
	Vps27	Ubiquitin and PI_3P binding. Recruits Vps23
	Hse1	Ubiquitin binding
ESCRT-I		
	Vps23	Ubiquitin binding. Interacts with Vps27 (ESCRT-0)
	Vps28	Interacts with Vps36 (ESCRT-II)
	Vps37	PI_3P binding
	Mvb12	Ubiquitin binding
ESCRT-II		
	Vps22	Non-specific membrane binding
	Vps25	Interacts with Vps20 (ESCRT-III)
	Vps36	Ubiquitin and PI_3P binding
ESCRT-III		
	Snf7	Forms long polymerized chains, interacts with Bro1 and Vps4
	Vps2	Interacts with Vps4
	Vps20	Interacts with Vps25 (ESCRT-II) and Vps4
	Vps24	Interacts with Did2
Vps4 complex		
	Vps4	AAA ATPase. Removal of ESCRT complexes
	Vta1	Promotes Vps4 oligomerization and ATP activity
ESCRT Associated Factors		
	Ist1	Negative regulator of Vps4
	Did2	Vps4 recruitment
	Bro1	Recruits Doa4. Interacts with Snf7
	Doa4	Deubiquitinating enzyme

(Henne et al., 2011; Katzmann et al., 2001). ESCRT-II triggers the polymerization reaction that results in the formation of ESCRT-III (Schmidt and Teis, 2012). Both ESCRT-II and ESCRT-III are known to concentrate cargo and are involved in the membrane deformation required for ILV formation (Schmidt and Teis, 2012). After ESCRT-III oligomerization, cargos are deubiquitinated by Doa4, a deubiquitinating enzyme. Doa4 is recruited to the ESCRT-III lattice by interacting with the ESCRT-III-associated protein Bro1 (Adell and Teis, 2011). After cargo is deubiquitinated, the final step of ESCRT complex disassembly can proceed.

The final step of MVB formation is the disassembly of ESCRT complexes from the endosomal membrane and the scission event that forms an ILV. Disassembly is mediated by the Vps4 complex, a mechanoenzyme that uses ATP hydrolysis to physically remove the ESCRT complexes from the late endosome and recycle them back into the cytoplasm (Henne et al., 2011). It is worth noting that the ESCRT complexes and the Vps4 complex are involved in more than just the MVB pathway. In higher eukaryotes, the ESCRTs mediate the final membrane abscission step during cytokinesis (Schmidt and Teis, 2012). Also, they are hijacked by retroviruses, like HIV and Ebola, for the release of mature virus particles from infected cells (Schmidt and Teis, 2012). All ESCRT-mediated membrane fission events share a similar membrane topology in that the membrane deforms in an orientation away from the cytoplasm. This is interesting because it is a topology opposite to that in many other budding processes in the cell.

Following the formation of the mature MVB, the last trafficking step is fusion with the vacuole. Endosome-to-vacuole fusion is mediated by the 'Homotypic fusion and vacuole Protein Sorting' (HOPS) complex (Balderhaar and Ungermann, 2013). The HOPS complex binds to the late endosomal and vacuolar Rab protein Ypt7p, which tethers mature MVBs and vacuole (Hickey and Wickner, 2010). After tethering, the HOPS complex catalyzes membrane fusion by interacting with SNARE proteins at the fusion site (Balderhaar and Ungermann, 2013). Once fusion has taken place, the intraluminal vesicles are released into the lumen of the vacuole. Upon exposure to the hydrolytic enzymes housed in the vacuole, proteins and lipids are broken down into their basic building blocks, which are recycled back into the cytoplasm by vacuolar nutrient transporters for further use by the metabolic pathways of the cell.

Downregulation of Fur4

General regulation. Nutrient transporters can be targeted for degradation as a consequence of a cellular response. The best understood cellular response resulting in wholesale turnover of nutrient transporters is acute starvation (Jones et al., 2012). Starvation-induced degradation results in the recycling of amino acids through the degradation of nonessential integral membrane proteins, which is essential for new protein production during the early phase of starvation (Jones et al., 2012). The target of rapamycin (TOR) kinase, a serine/threonine kinase, is responsible for initiating this early starvation response. In the cell, there are two TOR complexes. TOR complex 1 (TORC1) is responsible for regulating cellular pathways that control ribosomal biogenesis, induce autophagy, and block

translation initiation (Wang and Proud, 2009). TOR complex 2 is involved primarily in regulating the cytoskeleton (Cybulski and Hall, 2009; Wang and Proud, 2009)

There are two ways to increase the recycling of amino acids through the vacuole during acute starvation: (1) increase the amount of transmembrane proteins undergoing endocytosis and (2) increase the flux of cargos through the MVB pathway. The starvation response initiated by TORC1 influences both of these processes. During starvation, TORC1 is rendered inactive, which results in activation of the protein kinase Npr1 (Babst and Odorizzi, 2013a; MacGurn et al., 2011). Active Npr1 directly phosphorylates both Rsp5 and the Rsp5 adaptor ART proteins (MacGurn et al., 2011). Though not clearly demonstrated, the result of this phosphorylation is expected to increase ubiquitination of substrate proteins, thereby resulting in the increased endocytosis of nutrient transporters seen during acute starvation (Jones et al., 2012).

The way TORC1 moderates the MVB pathway is from a secondary effect of translation attenuation. The Vps4 negative regulator, Ist1, has been observed to regulate the MVB pathway in a protein concentration-dependent way (Jones et al., 2012). (Refer to Table 1.2 for Vps4-associated proteins.) During acute starvation, Ist1 protein levels drop due to a lack of new protein synthesis (Jones et al., 2012). This decrease in Ist1 levels is predicted to result in the increase of Vps4 activity in the terminal step of ILV formation, hence increasing the flux through the MVB pathway. The increase in endocytosis and MVB flux is a short-term survival mechanism that allows the generation of much-needed amino acids

required for the induction of the long-term adaptation of autophagy (Babst and Odorizzi, 2013b).

Fur4-Specific regulation: *Substrate-induced downregulation*. It has been well documented that an excess of extracellular substrate results in downregulation of nutrient transporters. For example, Fur4 is efficiently removed from the plasma membrane and targeted for degradation upon addition of high concentrations of uracil to the medium (Seron et al., 1999). Substrate-dependent downregulation ensures that the cytoplasmic concentration of uracil never reaches toxic levels, and allows the uracil that is transported into the cell to be effectively utilized by the pyrimidine salvage pathway (Seron et al., 1999). Uracil-induced downregulation of Fur4 requires the activity of Rsp5, for ubiquitination, on either of two lysines residues located in the N-terminus of the protein. Upon ubiqutination, Fur4 is efficiently endocytosed and trafficked into the MVB pathway. The molecular regulation of Fur4 substrate-dependent downregulation is explored in detail in Chapter 2.

Not only does exogenous uracil result in Fur4 downregulation from the plasma membrane, but also results in the direct sorting of newly synthesized Fur4 from the trans-Golgi to the endosome, a route referred to as 'biosynthetic pathway.' Most cargoes of the biosynthetic pathway are hydrolases, enzymes whose functional home is in the vacuole. The packaging of Fur4 into vesicles trafficked toward the endosome requires ubiquitination of Fur4 by Rsp5 (Blondel et al., 2004). The trigger for Golgi localized Fur4 to be ubiquitinated by Rsp5 is the direct binding of cytoplasmic uracil to Fur4 (Blondel et al., 2004). This is an

important observation that reveals how the cell regulates Fur4 downregulation based on uracil levels in the cytoplasm. This phenomenon is explored in detail in Chapter 2.

The sorting of ubiquitinated Fur4 into vesicles destined for the endosome requires the function of the GGA proteins. GGA proteins are coat adaptor proteins that facilitate clathrin-mediated vesicle formation (Scott et al., 2004). GGA adaptor proteins facilitate the sorting of cargos into the biosynthetic pathway (Nakayama and Wakatsuki, 2003). GGA proteins bind ubiquitin through their GAT domain, and it has been proposed that ubiquitin binding is responsible for diverting ubiquitinated nutrient transporters, like Fur4, into the biosynthetic pathway (Scott et al., 2004).

Quality Control of Multispanning Transmembrane Proteins

ER quality control. Newly synthesized Fur4 must acquire a native tertiary structure before being permitted to undergo ER exit and further trafficking along the secretory pathway (Needham and Brodsky, 2013). If a native fold is not achieved in a timely manner, the ER has a specialized quality control system, termed ERAD (ER-associated degradation), that ensures the degradation of these unfolded proteins (Needham and Brodsky, 2013). ERAD can be separated into three distinct systems: luminal, cytoplasmic, and membrane-anchored ERAD (Sato et al., 2009). Both the luminal ERAD (ERAD-L) and cytoplasmic ERAD (ERAD-C) utilize soluble chaperones that recognize exposed hydrophobic domains within unfolded proteins (Carvalho et al., 2006; Sato et al., 2009).

ERAD-M is specific for multispanning membrane proteins with limited cytoplasmic and luminal domains, a group to which Fur4 belongs (Carvalho et al., 2006; Sato et al., 2009). Therefore, in this introduction, I will focus on ERAD-M. (Refer to Table 1.3 for ERAD-M associated proteins.)

ERAD-M requires the cellular process of ubiquitination. There are four basic steps that must be accomplished for ERAD-M to take place: (1) initial recognition of a misfolded substrate, (2) removal from the ER membrane, (3) polyubiquitination by ubiquitin ligases, and (4) recognition and degradation by the 26S proteasome (Needham and Brodsky, 2013).

ERAD-M requires the function of the HRD ubiquitin ligase complex for tagging terminally unfolded transmembrane proteins with polyubiquitin chains. The HRD ubiquitin ligase complex is a multimeric protein complex built around Hrd1, a multispanning ER membrane protein with a cytoplasmic C-terminus containing the commonplace RING-H2 domain observed in many E3 ligases (Bordallo et al., 1998). The remaining components of the HRD complex are Hrd3, Usa1, and Der1 (Gardner et al., 2000; Horn et al., 2009). (Refer to Table 1.3 for an overview of proteins involved in ERAD-M.)

Hrd3 is anchored within the ER membrane by a single transmembrane domain that facilitates the interaction with Hrd1 (Gardner et al., 2000). Hrd3 also contains a large C-terminal luminal domain required for the interaction between ER luminal chaperones and lectins that is utilized for detection of unfolded luminal proteins during ERAD-L (Gardner et al., 2000). Hrd3 plays an important

Table 1.3 Protein complexes associated with ERAD-M

Protein Complex	Protein	Function
HRD complex: ERAD-M	Hrd1	E3 ubiquitin ligase
	Hrd3	Stabilizes Hrd1
	Usa1	Scaffolding protein and regulator of Hrd1 degradation
	Der1	Associates with Usa1
HRD complex associated proteins	Ubc7	E2 ubiquitin-conjugating enzyme
	Cue1	Ubiquitin-binding protein. Recruits Ubc7 to HRD complex
	Ubx2	Links the HRD complex with the Cdc48 complex
Cdc48 complex	Cdc48	AAA ATPase involved in the mechanical removal of proteins from the ER
	Npl4	Aids in Cdc48 mediated dislocation of ERAD substrates
	Ufd1	Polyubiquitin binding protein that aids Cdc48 mediated dislocation of ERAD substrates

role in the stability of Hrd1 and has the added ability to translate information from the ER lumen to the ring domain of Hrd1 (Gardner et al., 2000). Hrd1 requires for stability the scaffolding protein Usa1, which is also required for the recruitment of Der1, a HRD subunit that is dispensable for ERAD-M (Horn et al., 2009).

To date, there is one proposed mechanism explaining how the HRD complex is able to recognize unfolded transmembrane proteins. Within the

transmembrane region of Hrd1 is a collection of highly conserved amino acids that were observed to be instrumental in the identification of ERAD-M substrates (Sato et al., 2009). These amino acids are predicted to perform hydrophilic scanning of transmembrane regions of target proteins. Unfolded transmembrane proteins are thought to expose hydrophilic amino acids, which are recognized by Hrd1 (Sato et al., 2009). Not only can Hrd1 sense aberrant transmembrane domains, it can also recognize and facilitate degradation of nascent polypeptides that are unable to efficiently disassociate from the translocon (Rubenstein et al., 2012). Once Hrd1 has identified a substrate, it facilitates ubiquitination leading to retrotranslocation and eventual degradation by the 26S proteasome (Sato et al., 2009).

Central to ERAD is retrotranslocation of substrates from the ER lumen and membrane to the cytoplasm to allow access to the ubiquitination and degradation machinery. The mechanical energy required to remove membrane-integrated proteins comes from the mechanoenzyme Cdc48 (Finley et al., 2012). The Cdc48 ERAD function requires a number of regulatory proteins. (Refer to Table 1.3.) Two of the best-studied regulators are Ufd1 and Npl4, both involved in ubiquitin binding and substrate recognition (Shcherbik and Haines, 2007). Ufd1 and Npl4 form a heterodimer that, when bound to Cdc48, completes a functional retrotranslocation machine (Bays and Hampton, 2002). Recruitment of the Cdc48-Ufd1-Npl4 protein complex to the HRD complex is achieved through interactions with the membrane-bound ER resident Ubx2 (Neuber et al., 2005; Schuberth and Buchberger, 2005). Ubx2 plays a critical role in the coordination

between the HRD complex, Cdc48-Ufd1-Npl4, and unfolded protein substrates (Schuberth and Buchberger, 2005). Once an ERAD-M substrate has been recognized, ubiquitinated, and removed from the ER membrane, it is degraded by the 26S proteasome.

Quality control past the ER. Plasma membrane integrity is paramount to cell viability. Multispanning integral membrane proteins represent a weak link between the external chaotic environment and the tightly controlled intracellular space. The flexibility of nutrient transporters that allows for substrate transport into the cell is the same protein flexibility that could cause unfolding due to damaging events experienced by the cell. Such unfolded proteins would be detrimental to the cell if they created a pore allowing for unregulated flux of particles into or out of the cell. Unregulated flux could also result in the collapse of proton or other ion gradients required for nutrient transport. Therefore, a quality control system is needed at the plasma membrane that efficiently recognizes and degrades damaged nutrient transporters. A proposed quality control system will be discussed at length in Chapter 2.

Little is known about quality control of nutrient transporters past the ER. Because Fur4 can redirected into the endocytic pathway at the Golgi in a substrate-induced manner, there could be other forms of regulation within the Golgi. To date, there are no data depicting what would occur if a multispanning transmembrane protein were to unfold at the Golgi.

Quality control at the plasma membrane is equally unknown. To date, there are two examples involving quality control of integral plasma membrane

proteins within the mammalian system. The most interesting is the human cystic fibrosis transmembrane conductance regulator (CFTR) ion channel. CFTR has a well-characterized mutation, deletion of phenylalanine-508 (ΔF508), that results in temperature-induced unfolding of the protein (Okiyoneda et al., 2010). CFTRΔF508 expressed in cells grown at a permissive temperature allows for proper secretory trafficking and plasma membrane localization, but an increase to the restrictive temperature results in efficient downregulation (Okiyoneda et al., 2010). The large cytoplasmic domains of CFTRΔF508, enable cytoplasmic chaperones and C-terminal Hsp70-interacting protein to interact with exposed hydrophobic regions, which facilitates ubiquitination (Okiyoneda et al., 2010). Upon ubiquitination, CFTRΔF508 is targeted for degradation in an ESCRT-dependent manner (Apaja et al., 2010; Okiyoneda et al., 2010). This type of peripheral plasma membrane quality control has also been observed utilizing a known transmembrane segment attached to a large cytoplasmic temperature-sensitive protein. The large cytoplasmic domains of each of these proteins effectively mimic an unfolded cytoplasmic protein, and are recognized by the cytoplasmic quality control system. These two stories involving integral plasma membrane proteins with large cytoplasmic domains do not address how a quality control system would functionally recognize a multispanning integral membrane protein with limited cytoplasmic regions. The following chapters will explore the fundamental questions about the underlying mechanism required for Rsp5-dependent ubiquitination and subsequent downregulation. The chapters will

discuss a proposed model of substrate-dependent downregulation and quality control of cell surface nutrient transporters.

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CHAPTER 2

QUALITY CONTROL AND SUBSTRATE-DEPENDENT DOWNREGULATION OF THE NUTRIENT TRANSPORTER FUR4

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Quality Control and Substrate-Dependent Downregulation of the Nutrient Transporter Fur4

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Upon exposure to stress conditions, unfolded cell-surface nutrient transporters are rapidly internalized and degraded via the multivesicular body (MVB) pathway. Similarly, high concentrations of nutrients result in the downregulation of the corresponding transporters. Our studies using the yeast transporter Fur4 revealed that substrate-induced downregulation and quality control utilize a common mechanism. This mechanism is based on a conformation-sensing domain, termed LID (loop interaction domain), that regulates site-specific ubiquitination (also known as degron). Conformational alterations in the transporter induced by unfolding or substrate binding are transmitted to the LID, rendering the degron accessible for ubiquitination by Rsp5. As a consequence, the transporter is rapidly degraded. We propose that the LID–degron system is a conserved, chaperone-independent mechanism responsible for conformation-induced downregulation of many cell-surface transporters under physiological and pathological conditions.

Key words: degron, endocytosis, MVB pathway, plasma membrane protein, protein degradation, protein trafficking, ubiquitin

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The levels of nutrient transporters at the plasma membrane are regulated by several mechanisms, including regulation at the level of protein synthesis and degradation. These regulatory systems ensure a balance between the uptake of nutrients from the environment and the requirement for these nutrients by the metabolism of the cell. The substrate-dependent regulation of transporters has been studied in detail, utilizing the yeast high-affinity uracil importer Fur4 (reviewed in 1). High uracil concentrations in the growth medium not only suppress the transcription of the *FUR4* gene, but also result in the degradation of both its mRNA and protein (2). Artificial maintenance of high Fur4 levels under these conditions has been shown to cause cellular accumulation of toxic levels of uracil, demonstrating the importance of Fur4 downregulation in the presence of high substrate concentrations (2,3). The substrate-induced degradation of Fur4 involves phosphorylation of a PEST-like sequence in the N-terminal region of the protein and the ubiquitination of two neighboring lysine residues by the ubiquitin (Ub) ligase Rsp5 (Figure 1A) (4–6). Although phosphorylation of the PEST region increases the efficiency of Fur4 downregulation, it is not essential for ubiquitination and degradation of the transporter. Ubiquitination of Fur4 causes its rapid internalization and subsequent delivery, via the multivesicular body (MVB) pathway, to the vacuole for degradation.

Although the general scheme of Fur4 downregulation has been elucidated, the precise mechanism that triggers substrate-dependent Fur4 degradation is not known. Studies of Fur4 and other related transporters indicated that the interaction of the substrate with the substrate-binding site of the transporter is responsible for rapid downregulation of the protein, suggesting that the transporter itself serves as a sensor for the nutrient concentration present. However, conflicting models have been proposed with regard to the mechanism of sensing. Some studies supported the notion that active transport is necessary to induce ubiquitination of transporters (7), whereas other data indicated that the concentration of cytoplasmic substrate is key for the downregulation (3,8). In both models, conformational changes of the transporter itself are proposed to trigger the degradation of the protein.

Fur4 belongs to the nucleobase:cation symporter-1 (NCS1) family of transporters and imports uracil by using the proton gradient across the yeast plasma membrane. Crystal structure analysis of the bacterial homolog Mhp1 gave detailed insights into the mechanism of substrate import by this group of transporters (9,10). Fur4 is composed of 12 transmembrane domains that facilitate substrate import described by an alternative access model. The ground state of the transporter is the outward-facing open conformation that is able to bind extracellular substrate. Upon binding of the substrate, the transporter changes to an outward-facing occluded and then to an inward-facing occluded state. Finally, the transporter releases its substrate into the cytoplasm, resulting in an inward-facing open conformation. Any of these conformational changes might be key to trigger substrate-induced downregulation of the transporter.

Because nutrient transporters are gateways between the extracellular and intracellular environment, the fidelity and specificity of transport activity is of upmost importance for cell survival. Therefore, quality control that ensures the proper function of cell-surface nutrient transporters has to be highly sensitive and efficient in recognizing folding

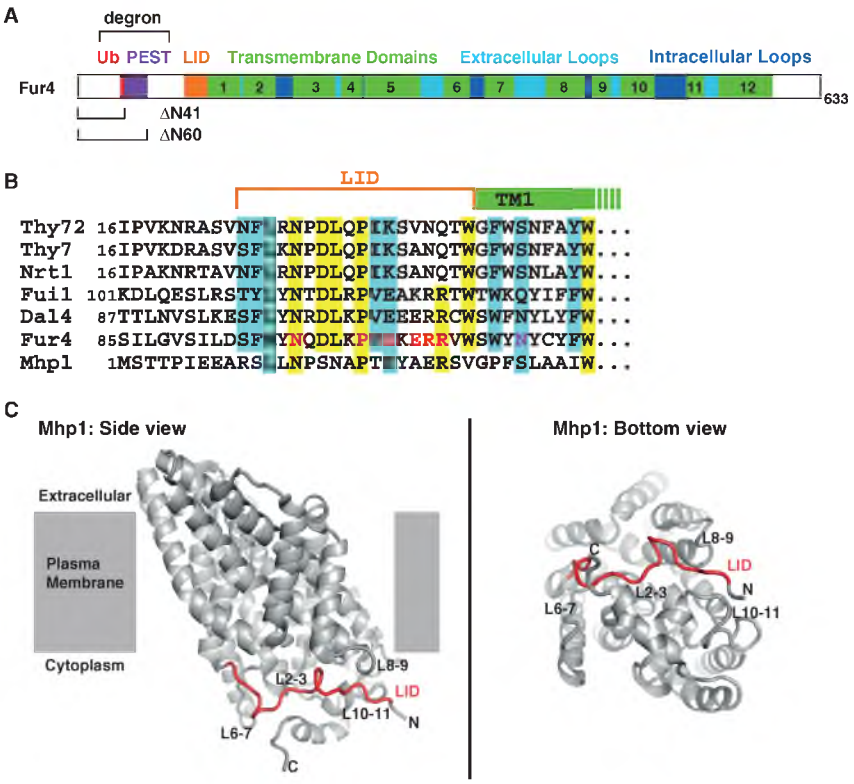

Figure 1: Structure of the transporters Fur4 and Mhp1. A) Schematic representation of Fur4 which contains 12 transmembrane domains. The N-terminally localized degron of Fur4 is composed of the ubiquitination site (Ub) and the PEST-like sequence, which is followed by the LID. Deletion of the first 60 amino acids removes the degron (ΔN60). B) Amino acid sequence alignment of the N-terminal regions of Fur4, Mhp1 and other homologous yeast transporters. Amino acids in Fur4 that have been mutated to alanine are labeled in red. The asparagine 115 of Fur4 is labeled in purple. C) Side and bottom view of the Mhp1 structure (based on crystal structure 2JLN). The LID is labeled in red and the cytoplasmic loops are indicated (L2-3, L6-7, L8-9, L10-11).

problems. It is well documented that environmental stresses that cause protein unfolding, such as heat shock or exposure to harmful chemicals, result in the rapid downregulation of cell-surface proteins, including Fur4, by a Ub-dependent mechanism (11).

The data presented in this study indicate that both substrate-dependent downregulation and quality control of nutrient transporters are mediated by the same intrinsic, conformation-sensing mechanisms. This mechanism is able to recognize deviations from the ground state of a transporter and trigger its ubiquitination and subsequent degradation.

Results

N-terminal domain is required for Fur4 quality control

Previous studies identified that phosphorylation and ubiquitination sites in the N-terminus of Fur4 are required for the rapid substrate-dependent degradation of the transporter (PEST and Ub in Figure 1A) (5). Deletion of the first 60 amino acids of Fur4 ($Fur4^{\Delta N60}$), which removes these N-terminal modification sites, results in stabilization of the transporter on the plasma membrane, even in the presence of high uracil concentrations (Figure 2A, lane #2). Surprisingly, the same deletion also inhibited rapid downregulation of Fur4-green fluorescent protein (GFP) at high temperature or in the presence of peroxide, conditions that are thought to induce conformational changes, resulting in a non-ground state or unfolded state of the protein. Whereas heat shock (1 h at 37°C) or peroxide treatment (0.005%, 30 min) of yeast cells resulted in the efficient delivery of wild-type Fur4-GFP to the vacuole for degradation, the mutant protein $Fur4^{\Delta N60}$-GFP remained at the plasma membrane (Figure 2A, lane #2). Uracil uptake assays demonstrated that peroxide treatment inhibited the uracil import of cells expressing $Fur4^{\Delta N60}$-GFP, supporting the idea that peroxide renders Fur4 non-functional (Figure 2B). However, shifting $Fur4^{\Delta N60}$-GFP expressing cells to 37°C did not inhibit uracil transport,

suggesting that these stress conditions are not severe enough to cause irreversible unfolding of Fur4 (data not shown). Together, these observations suggested that the quality control system, which is responsible for the rapid degradation of unfolded or partially unfolded Fur4, is dependent on the same N-terminal modifications that trigger the substrate-dependent downregulation. In particular, ubiquitination at K38 and K41 sites was found to be essential for Fur4 quality control, as mutating these sites completely abolished stress-induced Fur4-GFP downregulation (Fur4^{K38,41R}-GFP; Figure 2A, lane #3). The Ub ligase Rsp5 has been shown to be responsible for Fur4 ubiquitination at high substrate concentrations or at high temperature (6). Consistent with these previous reports, we observed that yeast strains expressing the mutant allele *rsp5-1* show no uracil- or stress-induced downregulation of Fur4-GFP (Figure 2A, lane #6). This result further supported the notion that Fur4 quality control is mediated by the same mechanism as substrate-dependent downregulation. However, in contrast to uracil-dependent downregulation, stress-induced degradation of Fur4 is not affected by the K272A mutation, a mutation that has been shown to block binding to uracil (Figure 2A, lane #4) (12). This result indicated that Fur4 quality control is independent of substrate binding.

To test if degradation of unfolded Fur4 requires the yeast cytoplasmic quality control system, we deleted two key Ub ligases, Ubr1 and San1, that have been shown to play an important role in the degradation of unfolded cytoplasmic proteins (13). In this mutant strain, the trafficking of Fur4-GFP was monitored after heat shock or peroxide treatment. Both stress treatments caused rapid downregulation of Fur4-GFP in the *ubr1*Δ*san1*Δ mutant cells indicating that the Ub ligases, Ubr1 and San1, are not required for stress-induced degradation of Fur4 (Figure 2A, lane #8).

Screen for Fur4 mutants that confer temperature-sensitive growth

Quality control of multispanning transmembrane proteins at the plasma membrane is predicted to play an essential role in maintaining the integrity of the cell. Unfolding of channels or transporters at the cell surface might cause an ion leak that could threaten the survival of the cell. To test this hypothesis, we took advantage of the mutant transporter Fur4$^{\Delta N60}$-GFP that is not downregulated under stress conditions and, thus, is predicted to remain in the plasma membrane even when unfolded. Low-fidelity polymerase chain reaction (PCR) was used to randomly mutagenize *fur4$^{\Delta N60}$-GFP*. The resulting mutant constructs were transformed into wild-type yeast and grown on plates at 25°C. The grown yeast colonies were then replica-plated and incubated at 37°C. After 2 days, yeast colonies were identified that lacked growth at 37°C. After re-testing the temperature-sensitive growth of the identified strains, one mutant strain was chosen for further analysis. The mutated *fur4$^{\Delta N60}$-GFP* gene was isolated and DNA sequence analysis identified a single base pair exchange at codon 115, causing an asparagine to histidine exchange in the first transmembrane domain of Fur4 (N115H; Figure 1B).

Growth tests showed that the expression of Fur4$^{\Delta N60,N115H}$-GFP not only impaired single colony growth at 37°C on plates (Figure 2C), but also inhibited growth in liquid medium at 30°C (Figure 2D). Osmotic support in the form of 1 M sorbitol suppressed the growth defect in liquid medium (Figure 2D). Mutations in the plasma membrane proton pump Pma1 are known to result in osmosensitive growth (14), suggesting that the observed growth phenotypes caused by the mutant Fur4 protein could be due to a proton leak across the plasma membrane. To test this idea, lysine 272 of Fur4$^{\Delta N60,N115H}$ was mutated to alanine. Lysine 272 is likely the proton carrier in Fur4, a prediction that is based on sequence comparison with the well-studied transporter Mhp1 and based on the observation that lysine 272 is the only charged amino acid within a transmembrane domain required for Fur4 activity (12). Cells expressing Fur4$^{\Delta N60,N115H,K272A}$ did exhibit only a weak osmosensitive growth phenotype, supporting the idea that a proton leak is the likely cause for the deleterious affects of Fur4$^{\Delta N60,N115H}$ (Figure 2D).

Both Fur4$^{\Delta N60}$ and Fur4$^{\Delta N60,N115H}$ are functional transporters at 25°C, as expression of each of these Fur4 mutants causes sensitivity to 5-fluorouracil, a toxic uracil homolog that is imported by Fur4 (Figure S1A). Consistent with this result, fluorescence microscopy showed normal plasma membrane localization of Fur4$^{\Delta N60,N115H}$ (Figure 2A, lane #5). Together, the data suggested that Fur4$^{\Delta N60,N115H}$ is a functional transporter at low temperature and unfolds when shifted to 37°C, causing the dramatic growth defect.

If the N-terminal region of Fur4 indeed functions in the quality control of the protein, we would predict that the N115H mutation in the context of the full-length Fur4 protein should cause rapid Fur4 degradation at high temperature. To test this prediction, wild-type Fur4-GFP and the N115H mutant form were transformed into yeast and the resulting strains were grown at 25°C. At exponential growth phase, cells were shifted to 37°C for 10 min and cells before and after temperature shift were analyzed by microscopy. In contrast to wild-type Fur4-GFP, which to a large extent remained at the plasma membrane, Fur4^{N115H}-GFP was rapidly internalized at 37°C, and a majority of the signal was found in endosomes (Figure 2E). Furthermore, cells expressing Fur4^{N115H} did not exhibit growth defects at high temperature or in liquid media (Figure 2C,D), indicating that the Fur4 quality control system was able to detect the temperature-induced folding problems in Fur4^{N115H}, trigger its rapid degradation and, thus, protect the cell from a potentially lethal ion leak. Similar to wild-type Fur4-GFP, rapid degradation of Fur4^{N115H}-GFP required Rsp5 (*rsp5-1*; Figure 2A, lane #7) but was independent of Ubr1 and San1, Ub ligases

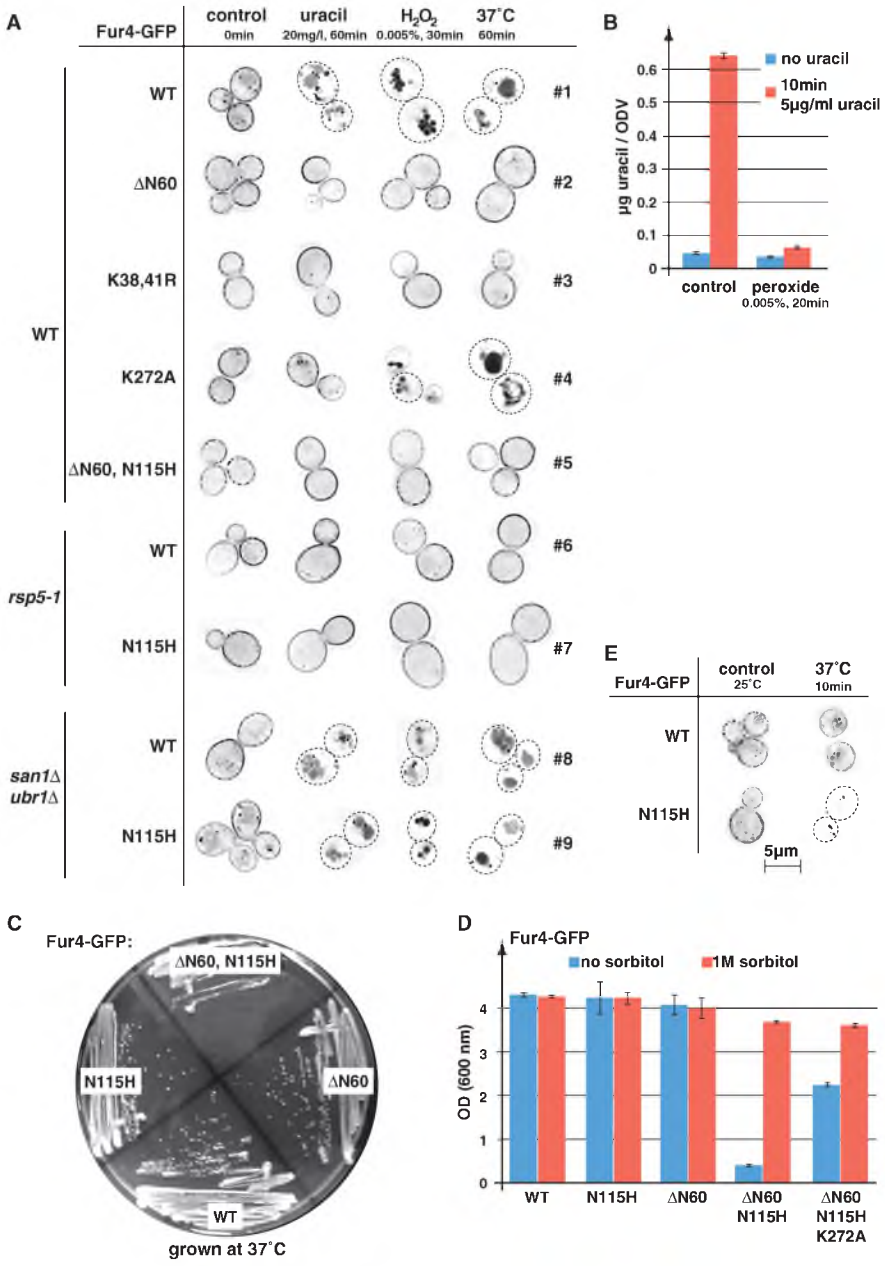

Figure 2: Stress-induced downregulation of Fur4 is dependent on the N-terminal degron. A) Fluorescence microscopy of yeast expressing wild-type and different mutant forms of Fur4-GFP, before and after treatment with uracil, peroxide or heat shock. B) Intracellular uracil concentration in cells expressing Fur4$^{\Delta N60}$-GFP. Cells were either not treated or treated with peroxide for 20 min and uracil concentration was determined before and after addition of 5 μg/mL uracil to the medium. C) Growth at 37°C of *fur4*Δ strains containing plasmids that express either wild-type or mutant forms of *FUR4-GFP*. D) Growth of *fur4*Δ strains expressing wild-type or different mutant forms of Fur4-GFP in liquid medium (YNB) at 30°C in the presence or absence of 1 M sorbitol. The graph represents the average growth of three cultures. E) Downregulation of wild-type and N115H mutant of Fur4-GFP after a 10-min heat shock.

involved in the cytoplasmic quality control (*san1*Δ *ubr1*Δ; Figure 2A, lane #9).

Substrate-dependent downregulation

Our data suggested that quality control of Fur4 requires the same ubiquitination event that has been shown to trigger downregulation in the presence of high uracil concentrations. Substrate-dependent downregulation is common among many cell-surface transporters. However, the precise mechanism of this induced degradation remains elusive. On the basis of the observation that the uracil-binding site in Fur4 is involved in sensing high substrate concentrations, it has been proposed that conformational changes that occur during pumping of the substrate might trigger ubiquitination. Alternatively, a model has been proposed in which a high cytoplasmic uracil concentration is the signal for Fur4 degradation (3). To test these models, we performed a systematic analysis of substrate-dependent Fur4 downregulation.

Extracellular substrate causes Fur4 downregulation

Because Fur4 efficiently imports uracil, adding it to the growth medium increases both the extracellular and cytoplasmic concentrations, making it impossible to differentiate between Fur4 downregulation triggered by intracellular or extracellular substrate. Therefore, we used the K272A mutation in Fur4, which has been shown to inhibit both binding and transport of uracil (12). Consistent with previous studies, we observed no uracil-induced downregulation of Fur4(K272A)-GFP. In contrast, upon addition of 20 mg/L uracil, wild-type Fur4-GFP was rapidly endocytosed and delivered to the vacuole for degradation (Figure 3A).

The immunosuppressant drug leflunomide is transported to the cytosol by Fur4 where it inhibits growth, possibly by blocking pyrimidine synthesis (15). This toxic effect of leflunomide is not observed when cells express Fur4(K272A)-GFP in a *FUR4* deletion strain, indicating that this mutant form of Fur4 is not only impaired in uracil import but also unable to transport leflunomide into the cell (Figure 3B). Interestingly, we observed leflunomide-induced downregulation of both wild-type Fur4 and Fur4(K272A), suggesting that, unlike uracil, leflunomide is able to efficiently bind to the K272A mutant of Fur4 (Figure 3A). Similar treatment of yeast expressing the methionine transporter Mup1-GFP showed no downregulation of this permease, demonstrating that leflunomide did not cause a general increase of endocytosis but specifically induced downregulation of Fur4 (Figure S1D). The lysine residues, K38 and K41, of Fur4 are targeted for ubiquitination in the presence of high uracil concentrations, a modification that is essential for uracil-dependent downregulation (5). Mutating these two lysine residues to arginine stabilized Fur4 not only in the presence of high uracil but also in the presence of leflunomide (Figure 3A), indicating that uracil and leflunomide trigger the same downregulation mechanism in Fur4.

Together, the data suggested that even in the absence of pump activity leflunomide is able to bind to Fur4 and induce its rapid downregulation. This observation further suggested that the switch of Fur4 from the outward-open or ground state to the outward-occluded conformation is sufficient to trigger its ubiquitination and degradation.

Cytoplasmic uracil causes Fur4 downregulation

Previous studies have observed that high uracil concentrations can redirect the trafficking of newly synthesized Fur4 at the *trans*-Golgi, resulting in the rapid degradation of the transporter in the vacuole (3). This result suggested that Fur4 downregulation is not induced by uracil transport but by the binding of cytoplasmic uracil to the transporter. To test if this model is correct for plasma membrane localized Fur4, we constructed two strains that would allow us to increase or decrease cytosolic uracil without adding uracil to the growth medium. The first strain constructed was deleted for the cytidine-deaminase gene *CDD1* (*cdd1*Δ), and the cytosine-deaminase gene was overexpressed with the help of a high-copy plasmid (2μ*FCY1*). These genetic modifications were expected to allow for efficient conversion of cytosine to uracil and vice versa (Figure 3C). The second strain overexpressed uracil-phosphoribosyltransferase (2μ*FUR1*), which was expected to cause rapid conversion of uracil to UMP, thereby lowering cytosolic uracil concentration (Figure 3C). To observe the trafficking of Fur4, wild-type and the two modified yeast strains were transformed with a plasmid expressing Fur4-GFP. Because some of the effects on Fur4 localization were less dramatic than observed in other experiments, 50 cells were analyzed for each condition and the ratio of internal signal (total signal minus plasma membrane signal) versus total signal was determined. The histogram in Figure 3E shows the distribution of these ratios for the three different yeast strains. Because of the cytoplasmic background, the intracellular/total ratios are larger than expected based on the microscopy pictures. For example, the wild-type control shown in Figure 3D has a ratio of 0.42, whereas the uracil-treated sample of the same strain in Figure 3D has a ratio of 0.86. Analysis of these data sets using the Kolmogorov–Smirnov test showed that all discussed differences are statistically relevant.

For the experiments, the yeast strains were grown to exponential phase in minimal synthetic medium lacking uracil and cytosine. Fluorescence microscopy demonstrated the expected localization of the majority of Fur4-GFP to the plasma membrane in all the three strains (Figure 3D). However, the quantitative analysis revealed a shift to a lower intracellular/total ratio for the *FUR1* overexpressing strain (2μ*FUR1*; Figure 3E), suggesting that the rapid conversion of uracil to UMP in this strain caused a stabilization of Fur4 on the plasma membrane, more so than the absence of extracellular uracil alone.

As expected, the addition of 5 mg/L uracil to the three different strains resulted in the rapid endocytosis and

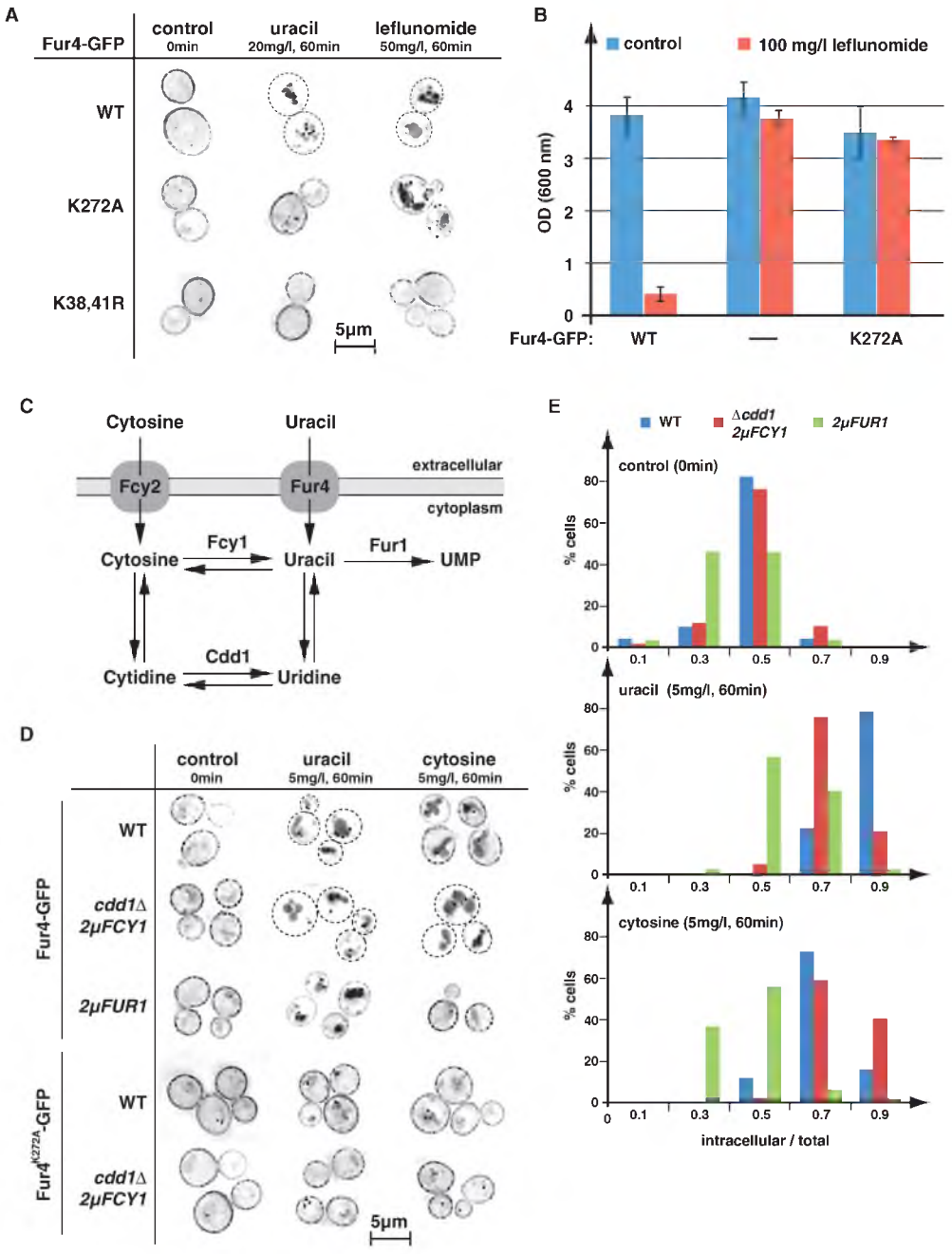

Figure 3: Extracellular and intracellular substrate initiates downregulation of Fur4. A) Downregulation of wild-type (WT) and mutant (K272A; K38,41R) Fur4-GFP in the presence of uracil or leflunomide. The fluorescence microscopy pictures are inverted and thus black indicates the localization of GFP. Dashed lines outline cells with no discernible plasma membrane signal. B) Optical density (OD 600nm) of yeast cultures grown overnight in the presence or absence of leflunomide. Yeast used for the experiment were deleted for Fur4 and transformed either with empty vector (–) or plasmids expressing either wild-type or the K272A mutant of Fur4-GFP. The results show the average growth of three cultures. C) Schematic representation of the uracil and cytosine metabolism of yeast. D) Uracil- and cytosine-induced downregulation of wild-type and K272A mutant of Fur4-GFP expressed in either WT, Δ*cdd1-2µFCY1* or *2µFUR1* strains. E) Quantification of the fluorescence microscopy shown in (D) (50 cells were quantified for each experiment). The graph shows the percentile of cells with a particular range of internal-to-total GFP signal (0.0–0.2, 0.2–0.4 and so on).

delivery of Fur4-GFP to the vacuole for degradation (Figure 3D). However, the degree of Fur4 downregulation was reduced in both the *cdd1*Δ-*2*μ*FCY1* and the *2*μ*FUR1* strains (Figure 3E). This result suggested that the conversion of cytosolic uracil either to cytosine or to UMP, respectively, stabilized Fur4 on the plasma membrane.

Yeast cells import cytosine via the Fcy2 transporter (Figure 3C). Previous studies have demonstrated that very high concentrations of cytosine in the growth medium induce Fur4 internalization, possibly caused by cytoplasmic uracil that was converted from imported cytosine (2). However, because of the high cytosine concentration used (40–60 mg/L), the study was not able to exclude the possibility that the converted uracil was exported from cells and re-imported, thereby causing the downregulation of Fur4. Therefore, for our experiments, we used low cytosine concentrations (5 mg/L) and quantified the effects on Fur4-GFP trafficking in our modified strains. In the wild-type strain, the addition of cytosine resulted in partial Fur4-GFP downregulation, an effect that was suppressed by the overexpression of *FUR1* (Figure 3D,E). In contrast, the presence of cytosine caused efficient Fur4-GFP downregulation in the *cdd1*Δ-*2*μ*FCY1* strain (Figure 3D,E). This result suggested that the imported cytosine was efficiently converted into uracil in the *cdd1*Δ-*2*μ*FCY1* strain, thereby triggering the degradation of Fur4. To ensure that the observed Fur4-GFP downregulation was not caused by extracellular uracil that was produced from cytosine and then exported from cells, a control experiment was performed in which uracil production and the Fur4-GFP reporter were separated into two strains. Wild-type cells expressing Fur4-GFP were mixed with *cdd1*Δ-*2*μ*FCY1* cells and the effect of cytosine addition was observed. In contrast to the previous experiment, where Fur4-GFP was present in the *cdd1*Δ-*2*μ*FCY1* cells, the addition of cytosine to the cell mixture did not cause Fur4-GFP downregulation (Figure S1E). Together, the results strongly supported a model in which Fur4 downregulation is caused by high uracil concentrations in the cytoplasm, indicating that uracil import activity of Fur4 is not required to trigger endocytosis of the transporter.

We observed that the K272A mutation impaired substrate-dependent downregulation of Fur4, even in experiments where uracil was intracellularly produced by conversion from cytosine (Figure 3D). This observation not only supported the idea that Fur4 itself is acting as a uracil sensor, but also indicated that uracil sensing was mediated by the Fur4 substrate-binding site. Similar observations were obtained in previous studies, which demonstrated the importance of the K272 site for substrate-dependent redirection of newly synthesized Fur4 at the *trans*-Golgi (3).

In summary, the analyses of substrate-dependent downregulation suggested that any substrate-bound state of Fur4 induces degradation of the transporter.

Table 1: Hydrogen bonds present between LID and the cytoplasmic loops in the ground state of Mhp1 (crystal structure 2JLN; hydrogen bonds missing in the structure 2X79 of the inward-facing occluded state of Mhp1 are marked; bb, backbone; sc, side chain)

LID		Loop			Distance	
Position	Atom	Position	Loop	Atom	(Å)	Not in 2X79
Arg 10	bb-O	Thr 397	10–11	bb-NH	3.2	X
Ser 11	bb-O	Arg 332	8–9	sc-NH	2.9	
Leu 12	bb-NH	Tyr 395	10–11	bb-O	2.9	X
Leu 13	bb-O	Arg 332	8–9	sc-NH	2.4	X
Asn 14	sc-NH_2	Pro 331	8–9	bb-O	2.6	X
Asn 17	bb-O	Tyr 395	10–11	sc-OH	2.9	
Thr 20	bb-NH	Gly 87	2–3	bb-O	3.2	
Arg 21	sc-NH	Arg 467	C-terminus	bb-O	2.6	
Arg 21	sc-NH	Asp 464	C-terminus	bb-O	3.0	X
Tyr 22	sc-OH	Arg 85	2–3	sc-NH	2.7	
Tyr 22	sc-OH	Glu 463	C-terminus	sc-O	3.1	
Arg 25	sc-NH	Cys 234	6–7	bb-O	3.2	
Arg 25	sc-NH	Ile 84	2–3	bb-O	2.6	
Arg 25	sc-NH	Gly 87	2–3	bb-NH	3.2	X
Ser 26	bb-NH	Glu 233	6–7	bb-O	3.2	
Val 27	bb-O	Lys 235	6–7	bb-NH	3.1	X

Downregulation of Fur4 is independent of transporter activity and can be triggered by binding of extracellular as well as intracellular substrate.

The Fur4 LID acts as a conformation sensor

Our substrate-dependent downregulation studies suggested that not a particular conformation but any substrate-bound state is able to trigger Fur4 degradation. This mechanism would explain how stress-induced unfolding of Fur4 causes downregulation by triggering the same ubiquitination as observed in the presence of high substrate concentrations: any Fur4 conformation that differs from the ground state of the transporter is targeted for degradation. If this model is correct, we would expect to find a domain in Fur4 that senses conformational changes and relays this information to the ubiquitination sites. To identify such a conformation-sensing domain, we studied the crystal structure of Mhp1, a bacterial homolog of Fur4 (Figure 1C). The Mhp1 structure showed that the ~20 amino acid region just prior to the first transmembrane domain is in an extended conformation and runs parallel to the membrane along a groove between the cytoplasmic loops (9). We call this N-terminal region as loop interaction domain (LID) (Figure 1A–C). In the outward-facing or ground state of Mhp1, the LID is kept in position by a series of hydrogen-bonding interactions with each of the cytoplasmic loops and the C-terminus (Table 1, Figure S2). Interestingly, about half of these interactions are lost when the transporter switches conformation to the inward-facing state (Table 1, Figure S3).

The structural information suggested that the LID of Mhp1 might stabilize the ground state of the transporter. Furthermore, the LID might function as the predicted

conformation sensor that could relay information about the functional state of the transporter to other cellular factors. We envisioned that such a mechanism could be responsible for inducing downregulation of Fur4, a homolog of Mhp1. This model was particularly attractive as the phosphorylation and ubiquitination sites necessary to trigger Fur4 degradation are located adjacent to the LID region of Fur4 (Figure 1A).

The amino acid sequence alignment of Fur4 with other NCS1-type transporters from yeast identified the predicted LID as a region with relatively high sequence conservation. In particular, a glutamine and a proline residue corresponding to the Fur4 positions 98 and 103, respectively, were identical in all sequences, including Mhp1 (Figure 1B).

Point mutagenesis was used to test if the predicted Fur4 LID and its loop interactions are involved in the downregulation of the transporter. The Mhp1 structure indicated that about half of the LID–loop hydrogen bonds were formed between protein backbone carbonyl and amino groups and are therefore not disrupted by changing the amino acid side chain (Table 1). However, the highly conserved glutamine at position 14 and the arginines at positions 21, 25 and 332 of Mhp1 formed hydrogen bonds mediated by their side chains. Thus, the corresponding positions in Fur4 were changed to alanines (red-labeled amino acids in Figure 1B). Three of these mutations were in the predicted LID region (N98A, E105A and R109A) and one mutation localized to loop 8–9 (K435A). In addition, the conserved proline residue of the Fur4 LID was mutated (P103A). The high conservation of this amino acid suggested that it might play an important structural role for the LID. As a control, two amino acids of the LID based on the Mhp1 structure that were predicted not to be involved in LID–loop interactions were also mutated (E107A and R108A).

The *fur4-GFP* mutant genes were expressed in wild-type cells and microscopy demonstrated that all mutant proteins localized properly to the plasma membrane. Growth tests in the presence of the toxic uracil analog 5-fluorouracil demonstrated that the mutant Fur4 proteins were functional transporters (Figure S1B,C). Furthermore, addition of uracil to the growth medium resulted in rapid downregulation of the mutant Fur4 proteins (Figure 4A). Together, the initial analysis of the Fur4 mutants suggested that these transporters function very similar to the wild-type protein. However, in fluorescence microscopy, Fur4(P103A)-GFP and Fur4(R109A)-GFP showed GFP signal surrounding the nucleus, which is reminiscent for endoplasmic reticulum (ER)-localized proteins (Figure 4A). This observation suggested that the P103A and R109A mutations affected folding of newly synthesized Fur4, resulting in an inefficient export from the ER. Therefore, as predicted from the Mhp1 structures, the LID–loop interactions seem to play an important role in stabilizing the basic fold of the transporter. Consistent with this idea,

Table 2: Analyzed Fur4 mutants and their phenotype

Fur4 Mutation	Corresponding position in Mhp1	Fur4 Stability (relative to WT)	
		Heat shock	Leflunomide
N98 to A	N14	↓	↓
P103 to A	P19	↓	↓
E105 to A	R21	↓	↓
E107 to A	A23	–	–
R108 to A	E24	–	–
R109 to A	R25	↓	↓
K435 to A	R332	↓	↓
M96BPA (+UV)	L12	↑	↑

we observed that N-terminal deletions that removed the Fur4 LID or regions close to the LID caused ER retention and degradation of the mutant Fur4 protein. GFP-tagged versions of these N-terminally deleted Fur4 proteins were barely detectable by fluorescence microscopy and the majority of the remaining signal localized to the ER (Fur4$^{\Delta N110}$-GFP and Fur4$^{\Delta N90}$-GFP; Figure 4B).

Our model predicts that the LID functions as a sensor, which is able to detect substrate- or stress-induced changes in the conformation of Fur4 and trigger the degradation of the transporter. If this model was correct, we would expect to observe increased downregulation of the mutant Fur4 proteins in the presence of low substrate or mild stress conditions. Therefore, cells expressing either wild-type or mutant Fur4-GFP were treated either with leflunomide or shifted to 37°C for 10 min. Leflunomide, and not uracil, was used for these experiments because this substrate is not metabolically converted and shows weaker affinity to Fur4, which increases the chance to observe differences in the sensitivity of different Fur4 mutants to the presence of substrate. Although both treatments resulted in downregulation of wild-type as well as mutant Fur4-GFP, the extent to which Fur4-GFP was endocytosed was much more pronounced in all mutant forms of Fur4-GFP that were predicted to have impaired LID–loop interactions (N98A, P103A, E105A, R109A and K435A; Table 2). Quantification of cells treated with leflunomide demonstrated that, dependent on the mutation, 18–100% of the mutant Fur4-GFP constructs showed no detectable plasma membrane signal, whereas almost all cells expressing wild-type Fur4-GFP or expressing the control mutant forms (E107A and R108A) retained some of the transporter at the cell surface (Figure 4A,C). Similarly, the 10-min heat shock resulted only in minor endocytosis of wild-type Fur4-GFP and the control mutants (E107A and R108A). In contrast, the same heat treatment caused the majority of the Fur4-GFP mutants predicted to carry destabilizing amino acid exchanges to localize to endosomal structures (Figure 4A, Table 2).

On the basis of our model, stabilizing the LID–loop interactions should decrease degradation of the transporter. To test this prediction, we used an amber-suppression

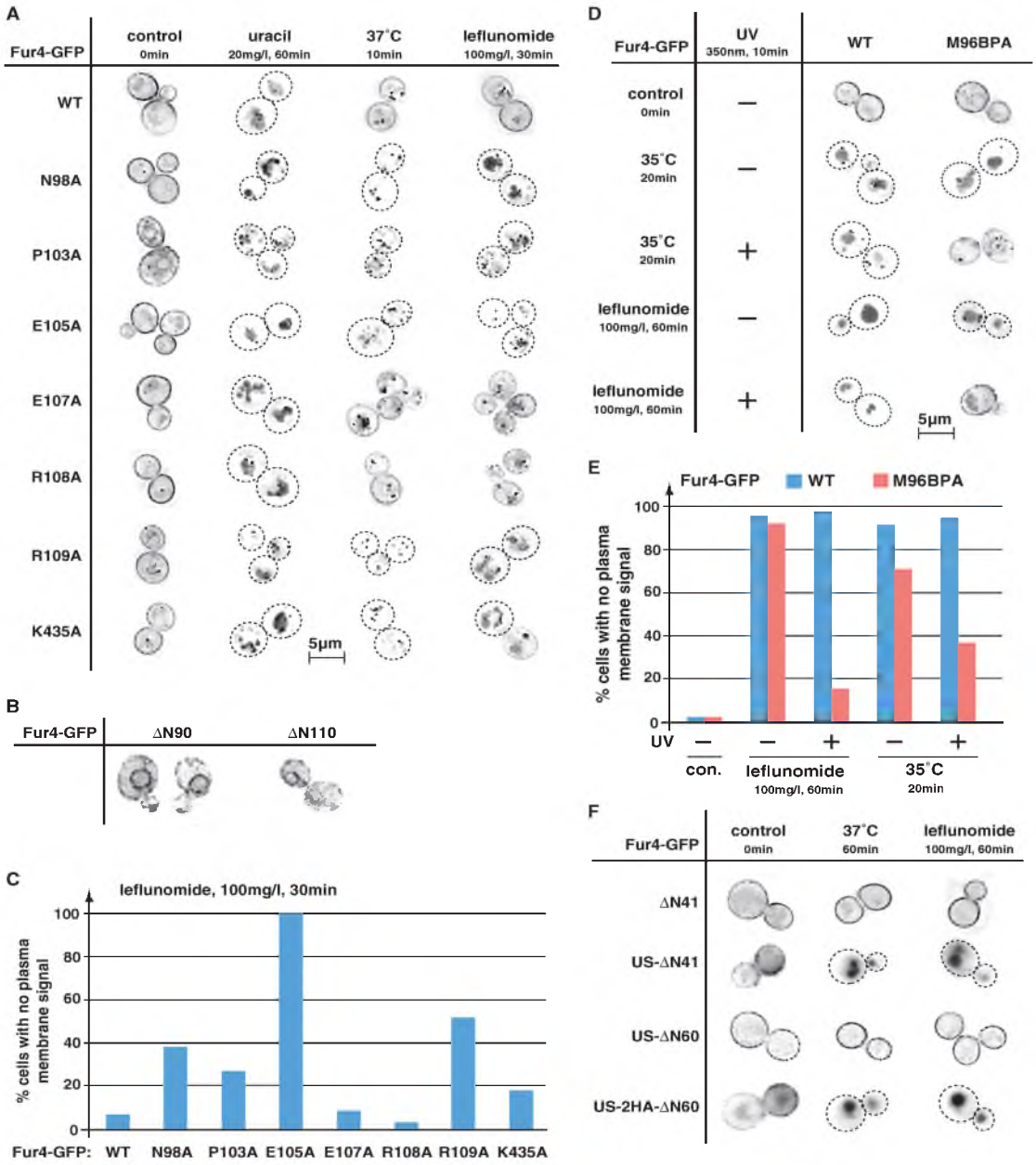

Figure 4: The LID regulates Fur4 degradation. A) Downregulation of wild-type and LID mutants of Fur4-GFP after treatment with uracil, high temperature or leflunomide. B) Deletion of the N-terminal 90 or 110 amino acids of Fur4-GFP resulted in ER retention and degradation of the protein. C) Quantification of the leflunomide treatment shown in (A). Approximately 30 cells were analyzed for the presence or absence of plasma membrane localized Fur4-GFP. D) Heat shock- and substrate-induced downregulation of wild-type Fur4-GFP and M96BPA mutant, before and after UV treatment. E) Quantification of the analysis shown in (D) ($N = 50$). F) Localization of different N-terminal mutants of Fur4-GFP before and after heat shock or leflunomide exposure.

system to change the methionine at position 96 of Fur4 to BPA (L-2-amino-3-(*p*-benzoylphenyl)propionic acid), an artificial photo-crosslinkable amino acid (16). This mutant was expressed in a yeast strain containing an amber suppressor t-RNA and its cognate aminoacyl-tRNA synthetase specific for BPA. The resulting Fur4(M96BPA)-GFP protein properly localized to the plasma membrane where it functioned in uracil import (Figures 4D and S1F). Upon UV exposure, BPA chemically crosslinks with other nearby molecules. On the basis of the Mhp1 structure, we expected that in UV-exposed Fur4(M96BPA)-GFP the BPA side chain would form covalent bonds to amino acids of the nearby cytoplasmic loop 10–11; however, crosslinking of BPA with lipids is also possible. As expected, we observed increased stability of Fur4(M96BPA)-GFP after UV treatment both in the presence of substrate (leflunomide) and stress conditions (heat shock; Figure 4D,E). The same UV treatment did not affect downregulation of wild-type Fur4-GFP, indicating that the UV-induced stabilization of the mutant transporter was dependent on the presence of BPA. The fact that UV treatment did not result in a complete block of Fur4(M96BPA)-GFP degradation might be explained by partial crosslinking of BPA to loop 10–11 and/or crosslinking to other molecules that do not restrict LID movements.

In summary, the phenotypes observed with the Fur4 mutants strongly supported the model that the LID functions in sensing conformational changes. Mutating loop–LID interactions mimics the loss of loop–LID interactions that normally occur as a result of substrate binding or unfolding of the transporter and, thus, the mutations decrease the stability of Fur4. In contrast, stabilizing the loop–LID interaction by chemical crosslinking caused increased stability of Fur4. Furthermore, the wild-type behavior of the control mutants E107A and R108A validated our approach to use the Mhp1 structure in designing the Fur4 mutants and demonstrated the high degree of structural conservation between these two transporters.

Fur4 ubiquitination is regulated by lysine 38, 41 accessibility

The data presented above suggested that loss of LID–loop interactions causes ubiquitination of the lysines at positions 38 and 41. The key question is: how does the LID regulate the degron? To gain insight into this regulation, we constructed a Fur4 mutant deleted for the first 41 amino acids, which removes the lysines targeted for ubiquitination. As expected, Fur4(ΔN41)-GFP localized to the plasma membrane even in the presence of substrate or stress conditions (Figure 4F). We then fused the ubiquitination site of Cps1, referred to as 'US' (amino acid sequence PVE*K*APRS), to the N-terminus of Fur4(ΔN41)-GFP. Cps1 is a transmembrane protein that is constitutively ubiquitinated by Rsp5 and traffics via the MVB pathway to the lumen of the vacuole (17). When expressed in yeast, Fur4(US-ΔN41)-GFP localized to the plasma membrane and, similar to the wild-type transporter, was internalized upon exposure to substrate or heat (Figure 4F). This result showed that the non-regulated Cps1 ubiquitination site was able to substitute for the deleted degron and restore regulated degradation, supporting the idea that regulation of ubiquitination is mediated by the LID.

The ubiquitination site US was also added to the N-terminus of Fur4(Δ60)-GFP, a deletion construct that remains on the plasma membrane even under stress conditions (Figure 2A). In contrast to Fur4(US-ΔN41)-GFP, Fur4(US-ΔN60)-GFP was not internalized upon heat shock or exposure to substrate (Figure 4F), suggesting that the amino acids between positions 41 and 60 play an important role in the ubiquitination of Fur4. To test if Fur4 ubiquitination depends on a particular amino acid sequence of the 41–60 region, we inserted a double HA tag (YPYDVPDYAYPYDVPDYA) downstream of the US sequence in Fur4(US-ΔN60)-GFP, thereby restoring the proper distance between the ubiquitination site and the LID. When expressed in yeast, the resulting construct Fur4(US-2HA-ΔN60)-GFP demonstrated substrate- and heat shock-induced downregulation of the transporter (Figure 4F).

Together, our observations suggested that substrate- or stress-dependent ubiquitination of Fur4 is independent of a particular amino acid sequence of the ubiquitination site or the neighboring regions but requires a certain distance between the LID and the lysines recognized by Rsp5. Therefore, we propose a model in which the LID regulates Rsp5's access to the ubiquitination sites. In the ground state of Fur4, the degron is 'tucked-in' and not accessible for Rsp5. However, the loss of loop–LID interactions that occur as a consequence of substrate binding or unfolding results in increased flexibility of the N-terminal region, which in turn allows Rsp5 to ubiquitinate the degron.

Mup1 quality control does not require Art1

Previous studies suggested that a group of proteins, known as the arrestin-related trafficking adaptors (ARTs), are responsible for quality control of cell-surface transporters (18). The ART proteins have been shown to bind to transporters and recruit Rsp5. No particular ART protein has been identified necessary for the downregulation of Fur4 (19). However, the methionine transporter Mup1, a member of the APC superfamily of transporters, has been shown to require Art1 for degradation (18).

We tested if quality control of Mup1 depends on the mechanism that is responsible for substrate-induced downregulation. As previously reported, high concentrations of methionine in the growth medium caused rapid Rsp5-dependent internalization of the transporter and its subsequent delivery to the vacuole for degradation (Figure 5) (20). Similarly, we observed that heat shock or peroxide treatment induced efficient downregulation of Mup1 in an Rsp5-dependent manner (Figure 5). Furthermore, stress-induced degradation of Mup1 was independent of Ubr1 and San1, the Ub ligases involved

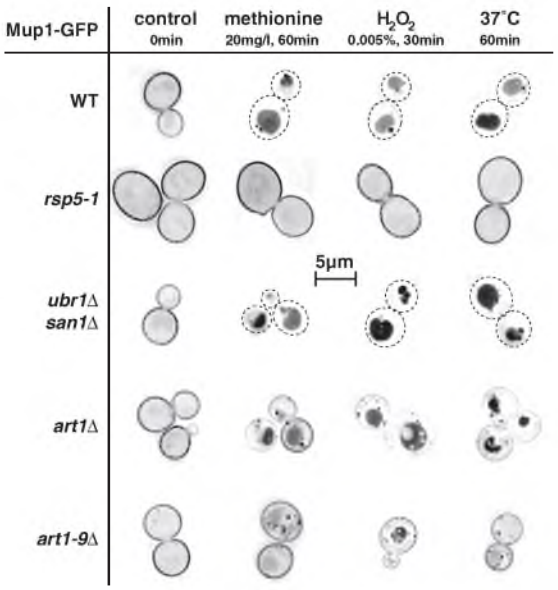

Figure 5: Quality control of Mup1 depends on Rsp5 but does not require Art1. Fluorescence microscopy of different Mup1-GFP expressing yeast strains before and after treatment with methionine, peroxide or heat shock.

in the quality control of cytoplasmic proteins (Figure 5). Together, these data were consistent with the Fur4 results and suggested that Mup1 quality control and methionine-induced downregulation were likely mediated by the same mechanism. However, in an *ART1* deleted strain, our fluorescence microscopy analysis showed a delay but not a block in the downregulation of Mup1-GFP triggered either by high substrate or stress conditions (*art1*Δ; Figure 5). This delay in the delivery of Mup1-GFP to the vacuole was more severe in a strain deleted for nine Art proteins (*art1-9*Δ; Figure 5), suggesting some redundancy among the Art proteins in the degradation of Mup1. Together, the data demonstrated that, in contrast to Rsp5, Art1 is not essential for substrate-dependent downregulation or quality control of Mup1 but rather seems to increase efficiency of Rsp5-dependent ubiquitination.

Discussion

Rapid degradation of cell-surface nutrient transporters is initiated either by cellular regulatory systems, such as the starvation response pathway (21), or by protein-specific events, including high substrate concentration or protein unfolding. On the basis of our studies of the yeast uracil transporter Fur4 and structural information from homologous bacterial transporters, we propose a model for the mechanism of protein-specific downregulation (Figure 6). The key element in this model is a cytoplasmic region of the transporter, referred to as LID , that interacts with intermembrane loop regions, thereby stabilizing the outward-facing or ground state of the transporter. Conformational changes in the transporter disrupt LID–loop interactions. The resulting increase in flexibility of the LID allows access to the degradation initiation site in the transporter, referred to as 'degron', that consequently is targeted for ubiquitination by plasma membrane localized Rsp5. The term degron is used to describe degradation signals that initiate the degradation of a protein in a controlled fashion (reviewed in 22). The ubiquitinated transporter is efficiently endocytosed and delivered via the MVB pathway to the lysosome/vacuole for degradation. In brief, this degradation model is composed of an intrinsic conformation sensor, the LID, that regulates a Ub site, the degron. The LID–degron system is highly versatile in that various deviations from the conformational ground state can trigger the degradation of the transporter, explaining how one mechanism can mediate both substrate-dependent downregulation and quality control of the protein.

Quality control of plasma membrane proteins is of vital importance for the cell as unfolded multispanning transmembrane proteins have the potential to form pores that compromise cell integrity. Therefore, an efficient system has to be in place that recognizes these unfolded proteins and initiates their rapid endocytosis and degradation. In the past, several studies have attempted to identify quality control factors that are essential for the rapid degradation of unfolded plasma membrane proteins (reviewed in 23). These studies found that mutations blocking endocytosis or the MVB pathway cause stabilization of the unfolded proteins. However, no specific quality control factors were identified. Two recent studies in mammalian cells identified a chaperon-mediated ubiquitination system that is responsible for the rapid turnover of unfolded plasma membrane proteins (24,25). This system is similar to cytoplasmic protein quality control, in that chaperones recognize the unfolded state of a protein and recruit the Ub ligase CHIP, which then marks the protein for degradation. However, both of these studies were based on membrane proteins containing large unfolded cytoplasmic domains. In these cases, a chaperon-based quality control system similar to that found in the cytoplasm is sensible. However, the question remained how chaperones would be able to recognize unfolded transmembrane regions, the type of folding problems that could lead to cell integrity problems.

The LID–degron system proposed by our study is able to explain how unfolding of transmembrane regions or extracellular domains triggers degradation of the transporter without the need of chaperones. This mechanism also explains why different protein unfolding conditions lead to the targeting of the same two lysines in the Fur4 degron, even though 15 other lysines are present within the cytoplasmic regions of the Fur4 protein. Deletion of the degron resulted in a block of Fur4 quality control at the plasma membrane. This lack of quality

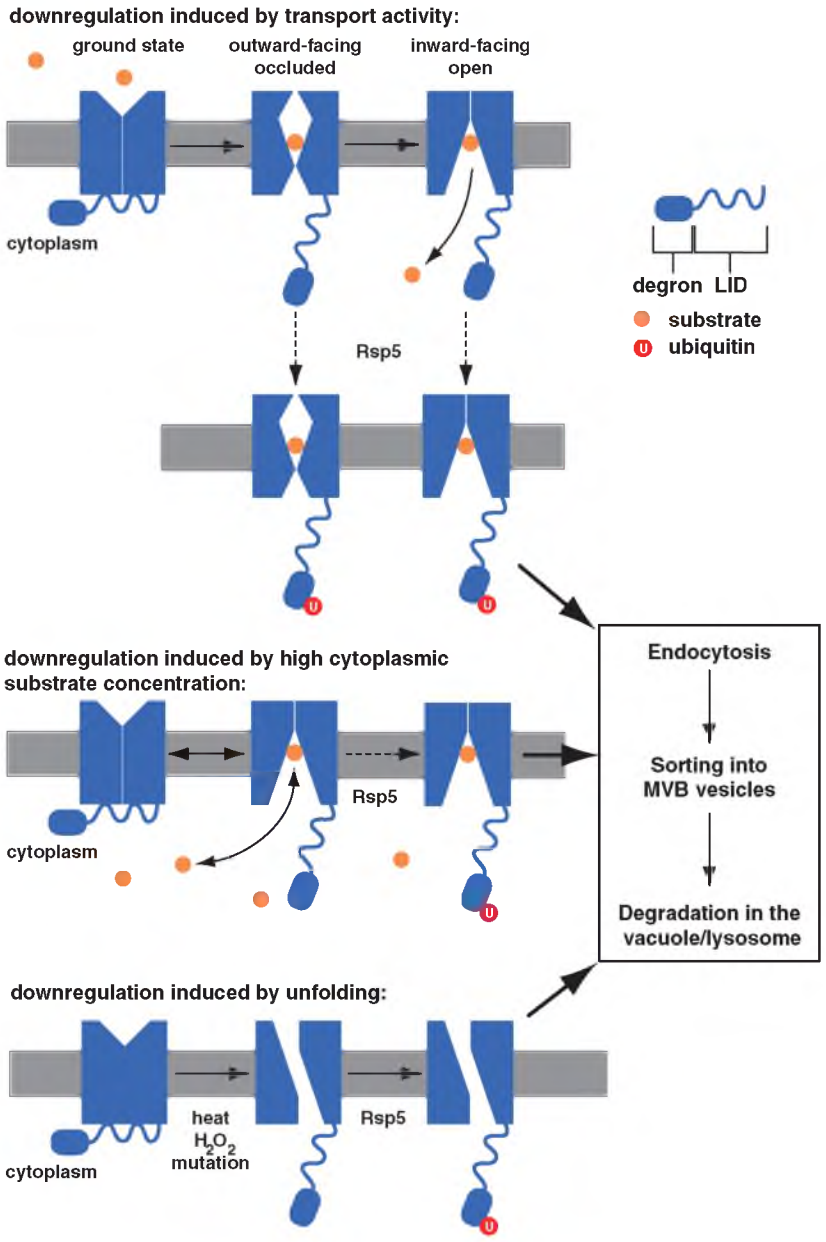

Figure 6: Model of substrate- and stress-induced Fur4 downregulation mediated by the LID–degron system.

control has the potential to cause severe damage to the cell, as demonstrated by the toxicity of a degron-deleted Fur4 containing a mutation in the first transmembrane domain (Figure 2). The expression of this mutant form of Fur4 caused severe growth defects, indicating that neither ER-localized nor cytoplasmic quality control was able to compensate for the lack of the LID–degron system.

Deletion of the LID caused ER retention and rapid degradation of Fur4 (Figure 4B), suggesting that the LID functions not only as a conformation sensor but also plays an important role for proper folding of Fur4. The LID–loop interactions might help arrange the transmembrane domains, thereby stabilizing the ground state of the transporter. This stabilizing role of the LID

would explain why the LID is conserved even in the transporters of bacteria, organisms that do not possess Ub-dependent degradation systems. The observation that LID deletions cause ER retention indicated that the ER quality control is independent of the LID–degron system and is able to recognize folding problems in the absence of the N-terminal region. Therefore, the LID–degron system seems to function in the Fur4 quality control past the ER, at the plasma membrane and possibly at Golgi and endosomal compartments.

Substrate-dependent degradation of nutrient transporters is an adaptation mechanism that is part of a regulatory system ensuring that proper number of transporters are present at the cell surface depending on the nutrient availability and cellular need. High substrate concentrations increase the turnover rate of transporters, whereas low substrate availability result in stabilization of the transporters. Previous studies found that the substrate-binding site in Fur4 is required for uracil-induced downregulation, suggesting that Fur4 itself is sensing the presence of uracil, thereby regulating its own turnover rate (2). Furthermore, we found that the presence of both extracelluar as well as intracellular substrate is able to induce internalization and degradation of Fur4 (Figure 3), suggesting that any substrate-bound state is able to trigger Fur4 ubiquitination. The LID–degron model fits well with these observations, which predicts that any major conformational change from the ground state of the transporter is sensed by the LID and can cause ubiquitination of the degron. However, our model does not predict that ubiquitination is an obligate step in the transport cycle of Fur4, rather that substrate-bound Fur4 has an increased chance of becoming ubiquitinated. Therefore, the critical parameter for ubiquitination efficiency is the time period of the substrate-bound state, which in turn depends on the uracil concentration. For example, at low cytoplasmic uracil concentrations, the substrate-bound conformations are short-lived because uracil is efficiently imported and released by Fur4, and, thus, the transporter remains mainly in the ground state. In contrast, high concentrations of cytoplasmic uracil will stabilize the inward-facing substrate-bound state, increasing the chance that the Fur4 degron is targeted by the Ub ligase Rsp5 (Figure 6). This type of regulation implies a coevolution of uracil-binding affinities of both Fur4 and the enzymes involved in the metabolism of uracil.

We predict that the LID–degron system is not unique to Fur4 but is conserved in a large number of transporters. Consistent with this prediction, we found that Mup1, a member of the APC transporter superfamily, showed Rsp5-dependent downregulation both under stress conditions and in the presence of high substrate concentrations. In contrast, the ART proteins, Rsp5 adaptors that have been previously suggested to function as Mup1 quality control factors (18), are not essential for stress- or substrate-dependent downregulation of Mup1 (Figure 5).

Members of APC superfamily include not only nutrient importers (e.g. Fur4 and Mup1) but also transporters of neurotransmitters, such as the serotonin transporter SERT, that play important roles in modulating neurotransmission in the brain. Structural studies of a bacterial homolog of SERT, known as LeuT, demonstrated the presence of several interactions between the N-terminus and cytoplasmic loops. These interactions are only observed in the ground state of LeuT but are lost as a consequence of substrate import (26). Therefore, SERT is likely to contain an LID–degron system similar to that of Fur4. The idea of an evolutionarily conserved LID–degron system is also supported by published studies of the amino acid transporter Gap1, which showed destabilization in mutants along the N-terminal region before the first transmembrane domain (27).

Materials and Methods

Yeast strains and plasmids

Saccharomyces cerevisiae strains and plasmids used in this work are described in Table 3. Genomic deletions of *FUR4* and *CDD1* were constructed by homologous recombination as previously described (28). All deletion strains were confirmed by PCR. Yeast strains were grown either in standard yeast extract-peptone-dextrose or, to maintain plasmids, in synthetic medium supplemented with essential amino acids as required (YNB) (29).

All *FUR4* clonings are based on the plasmid pFL38-FUR4-GFP (30). For growth assays, *FUR4-GFP* was expressed using the constitutive *SNF7* promoter. For microscopy, *FUR4-GFP* was expressed using the *CUP1* promoter that was induced with the addition of 0.1 mM cupric sulfate. Point mutations in *FUR4* were generated by site-directed mutagenesis with the Stratagene Quick Change kit (Agilent Technologies). DNA sequencing was used to confirm the mutations.

Fluorescence microscopy

Cells were grown to mid-log phase and analyzed by fluorescence microscopy using a deconvolution microscope (DeltaVision; Applied Precision). For experiments involving Mup1-GFP, cells were grown in minimal media lacking methionine. Quantification of the microscopy pictures was performed utilizing PHOTOSHOP software. Images of 50 random cells were deconvolved and saved as a projection in PHOTOSHOP format. Individual cells were selected and the boundary of any given cell was determined in the DAPI channel image with the wand selection tool. Total intensity of the whole cell as well as the intracellular region (cell outline contracted by 6 pixels) was recorded. For hydrogen peroxide treatment, cells were exposed to 0.005% H_2O_2 for 30 min, washed twice with PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH7.2) and resuspended in YNB media. Cells were allowed to recover for 30 min before microscopy was performed.

Uracil uptake assay

Cells were grown in minimal medium lacking uracil to mid-log phase. Uracil (5 mg/L) was added and the cells were harvested after 10 min, washed twice with ice-cold water and lysed in methanol at 50°C (5 min). The lysate was cleared twice by centrifugation (10 min, 20 000× ***g***) and the resulting supernatant was separated by high-performance liquid chromatography using a Luna-NH2 column (Phenomenex) in the presence of a 80–100% acetonitrile/water gradient. Uracil was detected at 260 nm.

Table 3: Strains and plasmids used in this study

Strain or plasmid	Descriptive name	Genotype or description	Reference or source
Strains			
SEY6210	WT	MATα *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	(31)
JKY5	*URA3*	SEY6210, *URA3*	This study
JKY6	*fur4Δ URA3*	SEY6210, *fur4::HIS5, URA3*	This study
JKY7	*cdd1Δ*	SEY62010, *cdd1::KanMX*	This study
JKY11	*mup1Δ*	SEY62010, *mup1::KanMX*	This study
RHY7450	*san1Δ ubr1Δ*	BY4741 *san1::NatMX ubr1::KanMX*	(13)
JPY88	*rsp5-1*	SEY6210 *rsp5::HIS3+pDsRED415-rsp5(G753I)*	(32)
MYY808	*rsp5-1*	MYY808 MATα, *MDM1, smm1, his3, leu2, ura3*	(33)
EN60	*art1-9Δ*	*ecm21::G418 csr2::G418 bsd2 rog3::natMX rod1 ygr068c aly2 aly1 ldb19 ylr392c::HIS*	(19)
JKY8	*art1Δ*	SEY6210, *art1::HIS5*	This study
Plasmids			
pPL4146	P(*CUP1*)-*MUP1-GFP*	*LEU2* (pRS315) P(*CUP1*)-*MUP1-GFP*	(34)
pJK19	P(*CUP1*)-*FUR4-GFP*	*URA3* (pRS416) P(*CUP1*)-*FUR4-GFP*	This study
pJK30	P(*CUP1*)-*fur4(Δ60)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(Δ60)-GFP*	This study
pJK28	P(*CUP1*)-*fur4(K272A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(K272A)-GFP*	This study
pJK31	P(*CUP1*)-*fur4(Δ60,N115H)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(Δ60,N115H)-GFP*	This study
pJK38	P(*CUP1*)-*fur4(N115H)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(N115H)-GFP*	This study
pJK25	P(*CUP1*)-*fur4(N98A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(N98A)-GFP*	This study
pJK26	P(*CUP1*)-*fur4(P103A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(P103A)-GFP*	This study
pJK27	P(*CUP1*)-*fur4(R109A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(R109A)-GFP*	This study
pJK29	P(*CUP1*)-*fur4(K435A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(K435A)-GFP*	This study
pJK12	P(*SNF7*)-*FUR4-GFP*	*TRP1* (pRS414) P(*SNF7*)-*FUR4-GFP*	This study
pJK20	P(*SNF7*)-*fur4(N98A)-GFP*	*TRP1* (pRS414) P(*SNF7*)-*fur4(N98A)-GFP*	This study
pJK21	P(*SNF7*)-*fur4(P103A)-GFP*	*TRP1* (pRS414) P(*SNF7*)-*fur4(P103A)-GFP*	This study
pJK22	P(*SNF7*)-*fur4(R109A)-GFP*	*TRP1* (pRS414) P(*SNF7*)-*fur4(R109A)-GFP*	This study
pJK24	P(*SNF7*)-*fur4(K435A)-GFP*	*TRP1* (pRS414) P(*SNF7*)-*fur4(K435A)-GFP*	This study
pJK32	P(*SNF7*)-*FUR4-GFP*	*LEU2* (pRS415) P(*SNF7*)-*FUR4-GFP*	This study
pJK34	P(*SNF7*)-*fur4(Δ60)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(Δ60)-GFP*	This study
pJK35	P(*SNF7*)-*fur4(Δ60,N115H)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(Δ60,N115H)-GFP*	This study
pJK33	P(*SNF7*)-*fur4(N115H)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(N115H)-GFP*	This study
pJK36	P(*SNF7*)-*fur4(K272A)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(K272A)-GFP*	This study
pJK39	P(*CUP1*)-*fur4(K38,41R)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(K38,41R)-GFP*	This study
pJK43	P(*CUP1*)-*fur4(E105A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(E105A)-GFP*	This study
pJk45	P(*SNF7*)-*fur4(Δ60,N115H,K272A)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(Δ60,N115H,K272A)-GFP*	This study
pJK50	P(*CUP1*)-*fur4(E107A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(E107A)-GFP*	This study
pJK47	P(*CUP1*)-*fur4(R108A)-GFP*	*URA3* (pRS416) P(*CUP1*)-*fur4(R108A)-GFP*	This study
pJK52	P(*SNF7*)-*fur4(E105A)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(E105A)-GFP*	This study
pJK51	P(*SNF7*)-*fur4(E107A)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(E107A)-GFP*	This study
pJK48	P(*SNF7*)-*fur4(R108A)-GFP*	*LEU2* (pRS415) P(*SNF7*)-*fur4(R108A)-GFP*	This study
pJK37	P(*FCY1*)-*FCY1*	*LEU2* (pRS425) P(*FCY1*)-*FCY1*	This study
pMB449	P(*FUR1*)-*FUR1*	*LEU2* (pRS425) P(*FUR1*)-*FUR1*	This study
pMB434	P(*SNF7*)-*fur4(ΔN110)-GFP*	*URA3* (pRS416) P(*SNF7*)-*fur4(Δ111)-GFP*	This study
pMB440	P(*SNF7*)-*fur4(ΔN90)-GFP*	*URA3* (pRS416) P(*SNF7*)-*fur4(Δ90)-GFP*	This study
pRS415	Empty vector		(35)
pRS414	Empty vector		(35)
pJK53	P*(CUP1)-fur4(M96BPA)-GFP*	LEU2 (pRS415) P*(CUP1)-fur4(M96BPA)-GFP*	This study
pJK54	P*(CUP1)-fur4(US-ΔN60)-GFP*	*URA3* (pRS416) P*(CUP1)-fur4(US-ΔN60)-GFP*	This study
pJK55	P*(CUP1)-fur4(US-2HA-ΔN60)-GFP*	*URA3* (pRS416) P*(CUP1)-fur4(US-2HA-ΔN60)-GFP*	This study
pJK56	P*(CUP1)-fur4(US-ΔN41)-GFP*	*URA3* (pRS416) P*(CUP1)-fur4(US-ΔN41)-GFP*	This study
pJK57	P*(CUP1)-fur4(ΔN41)-GFP*	*URA3* (pRS416) P*(CUP1)-fur4(ΔN41)-GFP*	This study

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The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Control experiments demonstrating functionality of Fur4 mutants and specificity of leflunomide treatment. A–C) Growth in presence or absence of 5-fluorouracil of fur4Δ strains containing either an empty vector (–) or plasmids expressing different versions of Fur4-GFP. Experiments shown in (A) and (C) used a different minimal medium than (B) (different auxotrophic selection). D) Control experiments testing that leflunomide does not induce downregulation of Mup1-GFP, (E) that uracil produced from cytosine in one cell does not induce downregulation of Fur4-GFP in another cell and (F) that Fur4(M96BPA)-GFP is able to efficiently import uracil from the growth medium.

Figure S2: LID-loop interactions in the ground state of Mhp1. LIGPLOT of the Mhp1 LID based on the crystal structure of the outward-open conformation (ground state, 2JLN).

Figure S3: LID-loop interactions in the substrate-bound state of Mhp1. LIGPLOT of the Mhp1 LID based on the crystal structure of the inward-occluded conformation (substrate-bound state, 2X79).

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CHAPTER 3

INVESTIGATION INTO MUP1 REGULATION AND FUNCTIONALITY

Introduction

The LID-degron model predicts that conformational changes in transporters induced by any means, either substrate or stress, should trigger degradation of the transporter. Therefore, downregulation should also be observed in the presence of small molecules that bind the transporter and affect its conformation and function. The best-studied small-molecule transporter inhibitors are the tricyclic antidepressants (TCAs), which block the human seretonin transporter SERT. SERT belongs to the APC family of transporters, a family that is closely related to the NCS1 transporters. TCAs block not only SERT but also other APC family members such as the bacterial amino acid transporter LeuT (reviewed in Nyola et al., 2010). Crystal structure analysis of TCA-bound LeuT indicated that TCAs are noncompetitive inhibitors of SERT and LeuT that act by blocking the transition from the outward-occluded to the inward-occluded state of the APC transporters (Zhou et al., 2007).

To further investigate the LID-degron model, the high affinity methionine transporter, Mup1, was utilized as a model cargo. Mup1, a member of the APC family of transporters, has been primarily studied as a model cargo for the

requirements of entry into the MVB pathway (Stringer and Piper, 2011). Like Fur4, Mup1 downregulation depends on ubiquitination by the activity of the E3 ligase Rsp5 (Lin et al., 2008). But unlike Fur4, Mup1 requires the arrestin-like adaptor protein Art1 for efficient downregulation (Keener and Babst, 2013; Lin et al., 2008). Based upon the LeuT-TCA crystal structure, TCA binding stabilizes a substrate-bound conformation, which is predicted by the LID-degron model to induce downregulation. It was explored if TCAs have the ability to inhibit function and induce downregulation of Mup1, the yeast homolog of LeuT, as predicted by the LID-degron. Mup1 trafficking was also investigated with respect to identifying the possible location of the LID domain and degron and Art1 binding site.

Materials and Methods

Saccharomyces cerevisiae strains and plamsids used are described in Table 3.1. *MUP1* genomic deletions were done using homologus recombination as previously described and confirmed by PCR (Sherman F, 1979).
Yeast strains were grown in knockout minimal medium to maintain plasmids (Longtine et al., 1998). Site-directed mutagenesis was performed with the Stratagene Quick Change kit (Agilent Technologies) and confirmed by DNA sequencing. The tricyclic antidepressants amitriptyline, clomipramine, desipramine, doxepin, and imipramine were purchased from Sigma-Aldrich.

Methionine uptake assay. Wild-type or *mup1*Δ cells were grown to mid-log phase in methionine dropout minimal medium. Cell cultures were diluted to a final concentration of OD_{600}=0.1 and pre-incubated for 5 minutes with 1.0 mM

Table 3.1. List of plasmids and strains used

Strain or plasmid	Descriptive name	Genotype or description	Reference or source
Strain			
SEY6210	WT	MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9	(Robinson et al., 1988)
JKY11	*mup1Δ*	SEY62010, *mup1::KanMX*	(Keener and Babst, 2013)
Plasmids			
pPL4146	P(*CUP1*)-*MUP1-GFP*	*LEU2* (pRS315) P(*CUP1*)-*MUP1-GFP*	(Stringer and Piper, 2011)
pJK60	P(CUP1)-*mup1*(K213A)-GFP	*LEU2* (pRS315) P(*CUP1*)-*mup1*(K213A)-*GFP*	This study
pMB446	P(SNF7)-*mup1(Δ30)*-GFP	TRP1 (pRS414) P(SNF7)-*mup1(Δ30)*-GFP	This study
pMB447	P(SNF7)-*mup1(Δ60)*-GFP	TRP1 (pRS414) P(SNF7)-*mup1(Δ60)*-GFP	This study
pEO73	P(SNF7)-Mup1-GFP	TRP1 (pRS414) P(SNF7)-Mup1-GFP	This study
pRS414	Empty Vector	TRP1 (pRS414)	(Christianson et al., 1992)
pRS415	Empty Vector	LEU2 (pRS415)	(Christianson et al., 1992)

amitriptyline hydrochloride or clomipramine hydrochloride. After pre-incubation, 10 nM of ^{35}S-methionine was added. Samples were collected at 0, 3, 6, and 9 minutes. Samples were collected with Milipore Durapore membrane filters and washed twice with 200 mM NaCl, 20 mM Tris pH8, and 1 mM methionine at 0°C. A finishing water wash was performed before samples were dried and analyzed in a scintillation counter.

Growth assay. *mup1*Δ cells expressing Mup1-GFP, *mup1(*Δ*30)*-GFP, *mup1(*Δ*60)*-GFP, mup1*(K213A)*-GFP, or an empty vector were grown to mid-log

phase in minimal medium lacking methionine with the addition of 0.1 mM cupric sulfate. Cells were added to either 0.1 mM cupric sulfate containing minimal media lacking methionine with the addition or absence of ethionine. 0.01 mM ethionine was used for P(CUP1)- Mup1 constructs and 1.0 mM ethionine was used for P(SNF7)-Mup1 constructs. Initial cultures were diluted to OD_{600}=0.025. Cultures were grown for 24 hours at 30°C and collected for OD measurements.

Fluorescence microscopy. Cells were grown to mid-log phase and imaged with a deconvolution microscope (DeltaVision; Applied Precision). Wild-type cells expressing Mup1-GFP, *mup1(Δ30)*-GFP, *mup1(Δ60)*-GFP, or mup1*(K213A)*-GFP were grown in minimal medium lacking methionine with 0.1 mM cupric sulfate. Substrate-dependent downregulation was induced by the addition of 20 ug/ml methionine for 1 hour. Photoshop was used for quantification of microscopy images. A total of 50 random cells were photographed and deconvolved and saved as a Photoshop document . Individual cells were analyzed for total cellular GFP intensity vs. intracellular GFP intensity. Data were recorded and analyzed in excel. For heat shock, 1.0 ml of mid-log phase cells were collected and incubated in a 37°C water bath. Hydrogen peroxide treatment was carried out by exposing cells to 0.005% H_2O_2 for 30 minutes at 30°C. Cells were then washed twice and suspended in minimal medium without methionine and allowed to recover for 30 minutes before microscopy.

Results

Mup1 is inhibited and downregulated by the tricyclic antidepressant drug clomipramine. Given that LeuT is stabilized in a substrate-bound conformation by

binding to TCAs, it was hypothesized that Mup1 would exhibit the same substrate-bound conformation if bound to TCAs. If, indeed, our LID-degron model (proposed in Chapter 2) is correct, then these drugs should trigger downregulation of the protein. To test this hypothesis, we analyzed the effect of five different TCAs on activity and trafficking of Mup1. The four drugs desipramine, imipramine, doxepin, and amitriptyline showed very little effect on Mup1 activity (amitriptyline Fig. 3.1 A; data for the other three TCAs not shown). In contrast, the addition of 1.0 mM clomipramine strongly inhibited Mup1-dependent uptake of methionine into yeast cells (Fig. 3.1 A). Furthermore, 1.0 mM clomipramine, but not 1mM amitriptyline, induced the downregulation of Mup1-GFP (Fig. 3.1 B). (The quantification of the total cellular fluorescence of 50 random cells for t=0, 1.0 mM clomipramine and 1.0 mM amitriptyline were analyzed for intracellular GFP vs. total GFP in Photoshop and are depicted in Fig. 3.1 C.) Clomipramine treatment had no effect on Fur4-GFP localization, indicating that clomipramine-induced downregulation of Mup1 was specific for this transporter (data not shown). These results suggested that clomipramine is able to bind and inhibit Mup1, most likely in a similar fashion as described for the LeuT-desipramine interaction. Most importantly, the binding of clomipramine induced the downregulation of Mup1, consistent with our NCS1 transporter model in which conformational changes are sensed by the LID and trigger ubiquitination and subsequent degradation of the transporter. (Refer to Fig. 3.2 for a model of TCA induced ubiquitination.)

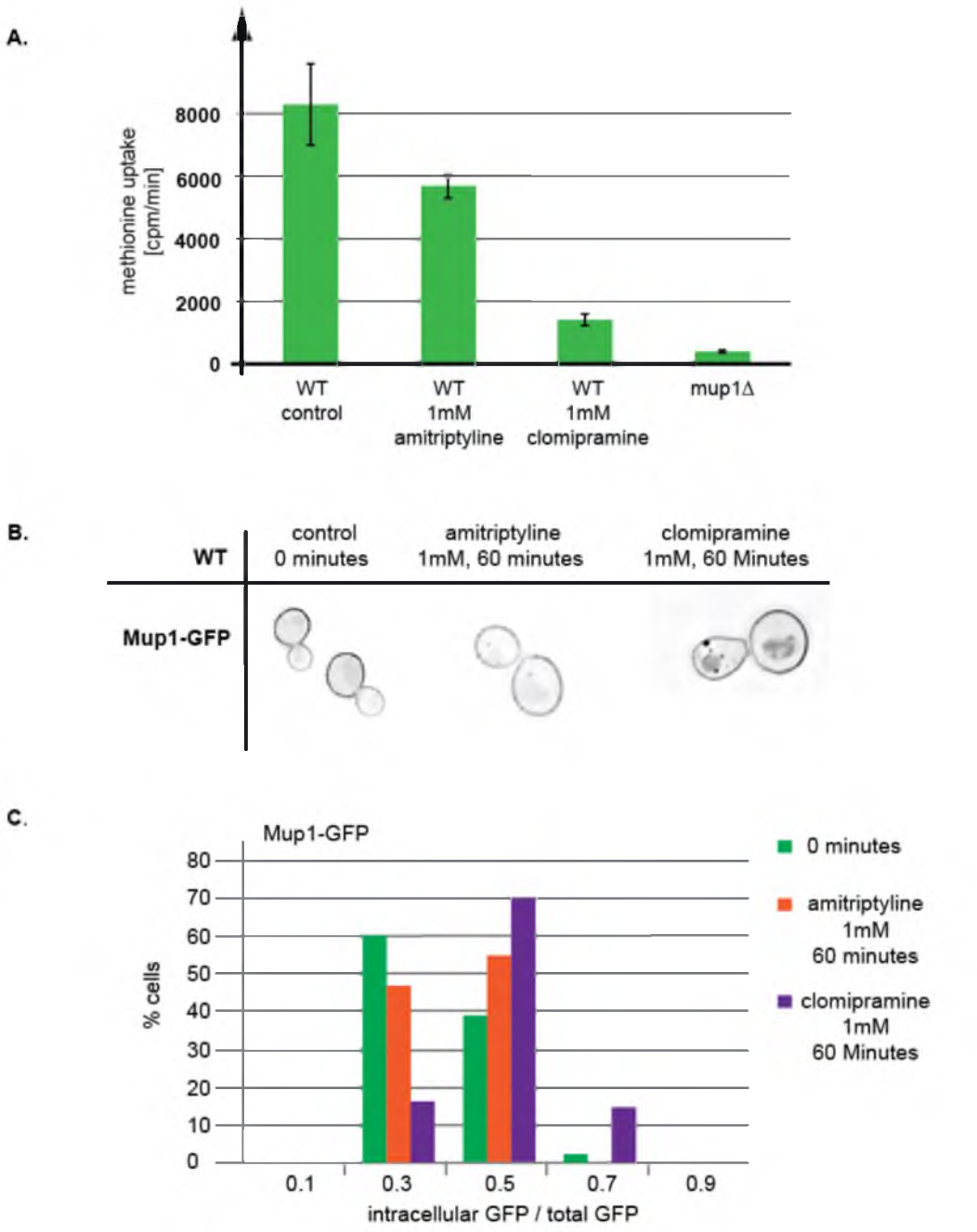

Figure 3.1 Mup1 downregulation and inhibition by clomipramine. A) Methionine uptake assay of wild-type yeast exposed to amitriptyline or clomipramine vs. mup1Δ cells. B) Downregulation of Mup1-GFP exposed to either amitriptyline or clomipramine. Fluorescence microscopy images are inverted. Black is the location of GFP. C) Quantification of the microscopy from B. (At least 50 cells were quantified for each experiment)

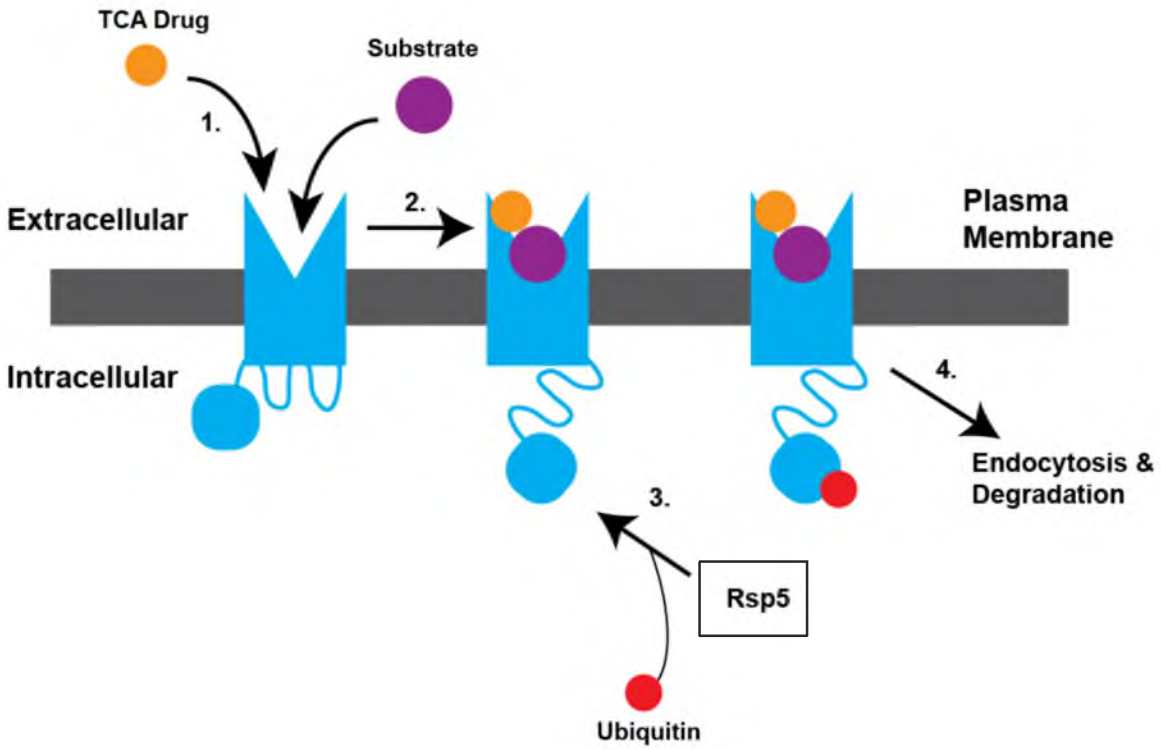

Figure 3. 2 Model of TCA drug-induced downregulation of Mup1

Predicted Mup1 N-terminus is dispensable for substrate- and stress-induced downregulation. Mup1 downregulation is facilitated by the ubiquitin E3 ligase Rsp5. Recently, Rsp5 has been described as utilizing a variety of adaptor proteins for the recognition of substrates. In the case of Mup1, the adaptor protein Art1 was necessary for endocytosis, but contradictory data has emerged for the role of Art1 (Keener and Babst, 2013; Lin et al., 2008). Recently, the ART-binding domains of two nutrient transporters were mapped, Lyp1, the lysine transporter, and Can1, the arginine transporter, both within their N-terminal region before the first transmembrane domain (Lin et al., 2008). To investigate if Mup1 has an N-terminal regulatory domain, deletion constructs were generated and assayed for functionality and trafficking. For identification of the predicted location of the first transmembrane domain, the primary amino acid sequence was examined with version 2.0 TMHMM hydrophobicity prediction software available from the Center for Biological Sequence Analysis. Amino acid 61 was predicted to be the start of the first-transmembrane domain. Two constructs were made, *mup1(Δ30)* and *mup1(Δ60)*, and were assayed for methionine-dependent downregulation. In the absence of extracellular methionine, both *mup1(Δ30)*-GFP and *mup1(Δ60)*-GFP localize to the plasma membrane like wild-type (Fig. 3.3 A). Surprisingly, the addition of 20 ug/ml of methionine after 1 hour resulted in both *mup1* constructs trafficking to the vacuole (Fig. 3.3 A). To confirm the transport activity of *mup1(Δ30)*-GFP and *mup1(Δ60)*-GFP, a growth assay was performed utilizing the methionine toxic analog ethionine (Colombani et al., 1975). Ethionine

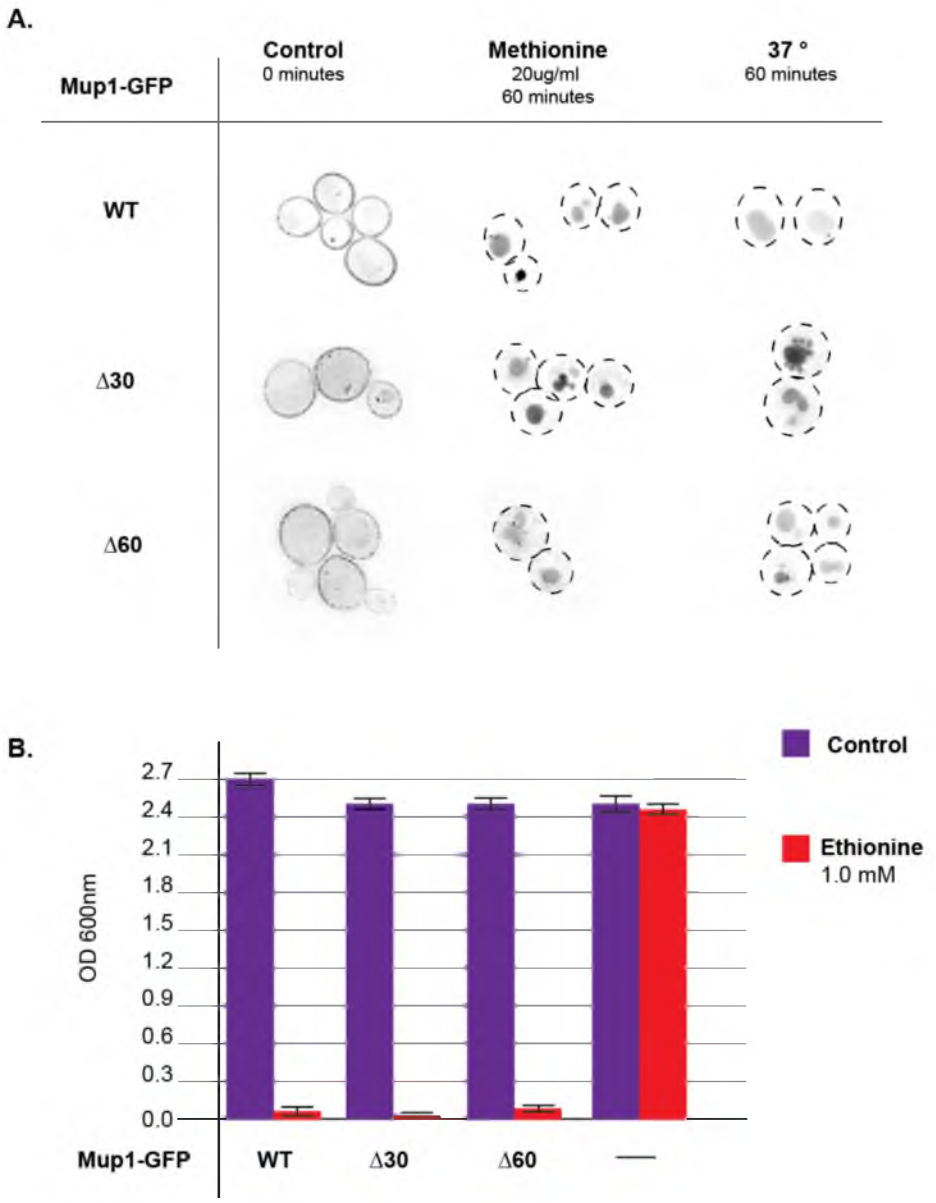

Figure 3.3 Mup1 N-terminal deletions do not block substrate- or stress-induced downregulation. A) Methionine- and heat stress-induced downregulation of Mup1-GFP and *mup1(Δ30)*-GFP and *mup1(Δ60)*-GFP expressed in WT. Dashed lines were added to cells if no discernable plasma membrane was observed. B) Optical density (OD 600 nm) of yeast cultures grown for 24 hours in the absence or presence of ethionine. Yeast used for this experiment were deleted for Mup1 and transformed with either empty vector (-) or plasmids expressing either Mup1-GFP, *mup1(Δ30)*-GFP, or *mup1(Δ60)*-GFP. Results demonstrate the average OD 600nm of three separate cultures.

is transported into yeast cells via Mup1 and results in death (Isnard et al., 1996). A *mup1Δ* yeast strain was transformed with constructs containing either Mup1-GFP, *mup1(Δ30)*-GFP, *mup1(Δ60)*-GFP, or an empty vector and placed in medium with or without 1.0 mM ethionine. Wild-type cells, as expected, were not able to grow in the presence of ethionine. Cells expressing *mup1(Δ30)*-GFP or *mup1(Δ60)*-GFP were also unable to grow, but *mup1Δ* containing an empty vector grew normally (Fig. 3.3 B). This result shows that the predicted N-terminus of Mup1 is not required for transport activity. Because it is expected that substrate-dependent downregulation is mechanistically the same as stress-dependent downregulation, as previously published for the uracil transporter Fur4, stress-dependent downregulation was tested (Keener and Babst, 2013). Both *mup1(Δ30)*-GFP and *mup1(Δ60)*-GFP-expressing cells were subjected to heat shock. Both fusion proteins were downregulated and delivered to the vacuole (Fig. 3.3 A).

K213 is required for Mup1 methionine transport and downregulation. It has been reported that charged residues within transmembrane domains help to stabilize the fold of a multispanning transmembrane proteins and are involved in their transport function (Pinson et al., 1999). Based upon the observation that Fur4 family members demonstrate highly conserved charged residues within important transmembrane domains, Mup1 family members were analyzed for highly conserved charged amino acid residues within transmembrane domains (Pinson et al., 1999). K213 was selected as a mutagenesis candidate. Amino acid replacement K213A was tested for functionality. Mup1-GFP was compared

to *mup1(K213A)*-GFP in methionine-induced downregulation. In the absence of methionine, both Mup1-GFP and *mup1(K213A)*-GFP were localized properly to the plasma membrane (Fig. 3.4 A). Methionine was then added to the cells and after a 60-minute incubation time, samples were taken for microscopy. The wild-type protein localized to the vacuole, indicative of substrate-induced downregulation, but *mup1(K213A)*-GFP remained at the plasma membrane (Fig. 3.4 A). To test if *mup1(K213A)* was a nonfunctional transporter, a growth assay was performed using the methionine toxic analog ethionine. A *mup1*Δ yeast strain was transformed with either empty vector, Mup1, or *mup1(K213A)* constructs and grown in the presence or absence of ethionine. Only cells expressing a functional ethionine transporter would not be able to survive. Mup1-expressing cells were unable to grow, whereas *mup1(K213A)* were able to grow (Fig. 3.4 B). This result demonstrates that *mup1(K213A)* is a nonfunctional transporter. Based on our studies of Fur4, we would predict that transporter function is not a prerequisite for stress-dependent downregulation (Keener and Babst, 2013). To test if this is true for Mup1, *mup1(K213A)* was subjected to peroxide treatment or heat shock. Both stressors resulted in efficient downregulation and vacuole trafficking of the mutant transporter (Fig. 3.4 A).

Discussion

The LID-degron system is an intrinsic conformational sensing system first discovered in the high affinity uracil transporter, Fur4. (Refer to Fig. 2.6 in

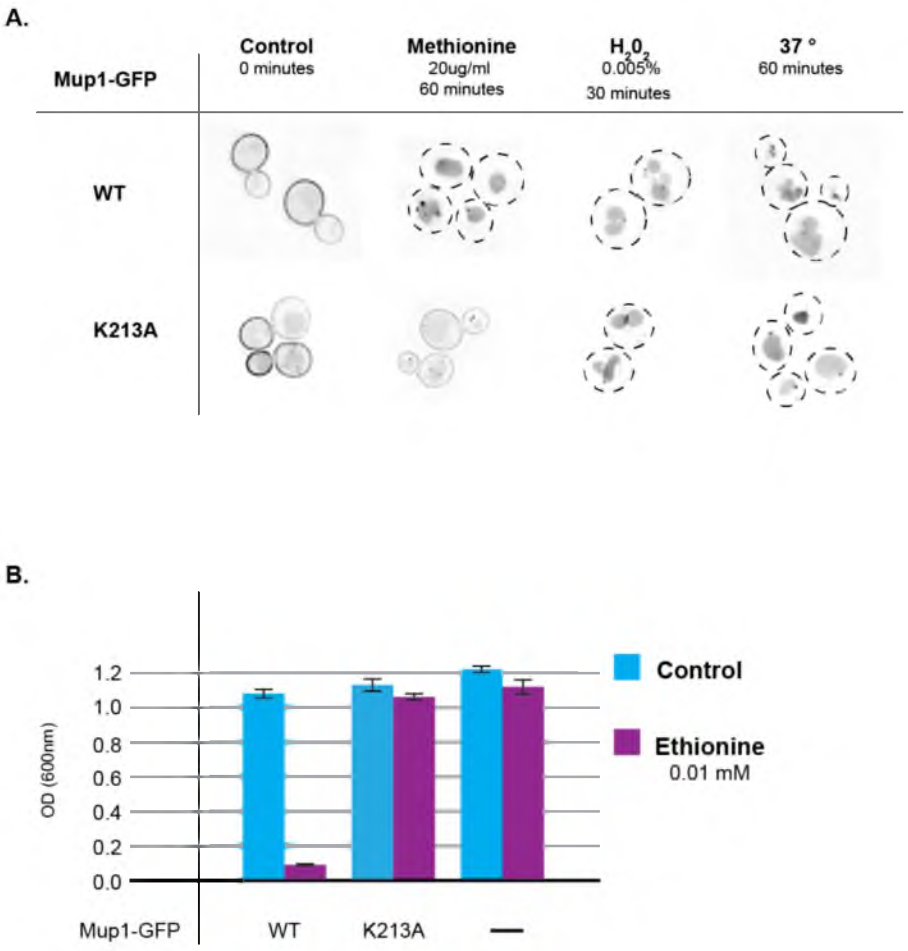

Figure 3.4 Mup1 K213 is required for substrate-induced downregulation but not for stress-induced downregulation. A) Downregulation of Mup1-GFP and *mup1(*K213A)-GFP after the addition of methionine or exposure to oxidation or heat stress. B) Growth assay of *mup1*Δ transformed with empty vector (-) or plasmids expressing Mup1-GFP or *mup1*(K213A)-GFP. Yeast cultures were either exposed to ethionine or not allowed to grow for 24 hours when optical density (OD 600nm) was measured. Results are an average of three separate cultures.

chapter two, page 50.) The LID domain senses the folding status of the protein through hydrogen bond interactions with the cytoplasmic loops of the protein. In the ground state, the LID makes all possible hydrogen bonding contacts with cytoplasmic loops, resulting in the sequestration of the degron. If the protein undergoes unfolding for any reason, such as substrate binding/transport or unfolding due to damage, the hydrogen bonds between the LID and cytoplasmic loops are broken, resulting in the presentation of the degron to cellular ubiquitination machinery. The LID-degron model explains why high substrate concentrations and protein damage both result in efficient downregulation.

The LID-degron system is most likely not unique to Fur4, but rather conserved within a large number of transporters. Fur4 is a member of the APC transporter superfamily. A well-studied member of this superfamily is LeuT, a bacterial leucine importer that serves as a model for the human transporter SERT. SERT is expressed in neurons of the brain where it functions in the re-uptake of serotonin. Antidepressant drugs, such as the tricyclic antidepressants (TCAs), impair SERT activity, which increases serotonin levels in the brain, thereby alleviating the symptoms of depression (reviewed in (Butler and Meegan, 2008)). Crystal structure analysis of TCAs bound to LeuT indicated that these drugs inhibit the import activity of the transporters by stabilizing a substrate-bound intermediate state of the transporter (Heck et al., 2010). Based on this information, we expected that TCA molecules should trigger the degradation of the TCA-bound transporter. Indeed, our studies of the yeast APC transporter Mup1 found that the TCA drug clomipramine not only inhibited the substrate

uptake but also induced downregulation of the transporter. (Refer to Fig. 3.2.) Consistent with our findings, studies in mice and rats have shown that TCAs cause a decrease in the levels of SERT in brains of the treated animals (Mirza et al., 2007; Nadgir and Malviya, 2008; Zhao et al., 2009).

Together, the results of the Mup1 analysis were consistent with the Fur4 data and suggested that the LID-degron mechanism of substrate- and stress-induced degradation is conserved among many transporters. (Refer to Fig. 2.6 and 3.2 for a schematic model of the LID-degron system.) Furthermore, our study of the TCAs demonstrated the potential for the development of new drugs that are not directed at blocking the import function of the transporter, but rather designed to induce conformational changes that cause the rapid downregulation of the target protein.

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CHAPTER 4

CONCLUDING REMARKS

Quality control of multispanning integral membrane proteins is essential for cell viability. The most studied membrane quality control system in the cell is ERAD-M. ERAD-M is able to recognize and target for degradation aberrantly folded membrane protein. Many facets of ERAD-M remain unknown. Past the ER, almost nothing is known about quality control of multispanning integral membrane proteins. The best example to date is that of the CFTRΔF508 protein, which displays quality control when located at the plasma membrane. The quality control system that recognizes the large unfolded cytoplasmic region of CFTRΔF508 is the cytoplasmic quality control system. The cytoplasmic quality control system relies on the activity of cytoplasmic chaperones for recognition of unfolded proteins and the recruitment of ubiquitin ligases required for ubiquitination and degradation. This quality control system requires a large cytoplasmic domain, which many nutrient transporters do not have.

The question that this work strived to answer was: How is the cell able to determine that a protein is unfolded if it is primarily imbedded in the membrane? The LID-degron system we uncovered explains how quality control of transmembrane proteins can occur without the need for cytoplasmic chaperones. (Refer to Fig. 2.6 in Chapter 2.) The LID-degron model is also able to explain

how Rsp5 can target specific proteins as substrates regardless of the reason for downregulation (stress or substrate).

The LID-degron model relies on the intrinsic sensing mechanism of the LID. In the ground state, the LID makes key hydrogen bonding contacts with cytoplasmic loops of the transporter, whereas in any nonground state, the LID is no longer able to make these hydrogen bonds. This change in LID conformation is relayed to the degron, which in turn recruits Rsp5 for ubiquitination. Ubiquitinated Fur4 is rapidly endocytosed and delivered to the vacuolar lumen for degradation.

The LID-degron model not only explains quality control-dependent downregulation of Fur4, but also substrate-induced downregulation of many nutrient transporters. Substrate transport and binding both result in protein conformations that deviate from the ground state. The time spent in a non-ground state is dependent upon the concentration of cytoplasmic substrate. This nonground state induced by substrate results in the same LID-degron mechanism of Rsp5 ubiqutination seen with protein unfolding.

The LID-degron mechanism functions in the absence of cytoplasmic chaperones or the E3 ligases, San1 or Ubr1, which are required for the cytoplasmic quality control system. Also, unlike the cytoplasmic quality control system, the LID-degron system relies on specific lysines for ubiquitination. The LID-degron model presents a shift in thinking about quality control, from chaperone-mediated, external quality control to an intrinsic system in which the protein monitors its own folding state. This model is most likely not only relevant

to Fur4, but could be applied to any multispanning cell surface protein with limited cytoplasmic regions. Although the investigation into Mup1, another APC transporter superfamily member, indicated that the N-terminal region of this transporter did not contain a LID-degron system, it is possible that the LID and degron motifs are present in one of the cytoplasmic loops or the C-terminus of Mup1.

The LID-degron system is a straightforward model that is able to explain how a multispanning integral plasma membrane protein with limited cytoplasmic regions can undergo quality control without the aid of cytoplasmic chaperones or an ERAD-M like system. Though the work presented in this thesis begins to fill in the unknowns of plasma membrane quality control, there are still many open questions in the field. For example: How conserved is the LID-degron mechanism? Do higher eukaryotes have proteins that exhibit the LID-degron system? What about bacterial proteins? And what would explain the evolution of the LID-degron system from bacteria to eukaryotes? The work presented here has laid the groundwork for others to further explore the LID-degron system.